Assessing the risk of fungal stem defects that affect sawlog quality in Vietnamese *Acacia* plantations

by

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Abstract

Acacia hybrid clones (*Acacia mangium* x *A. auriculiformis*) are widely planted in Vietnam. An increasing proportion of the *Acacia* hybrid plantations established (now standing at 400,000 ha) is managed for solid wood, mainly for furniture. Silvicultural practices such as pruning and thinning ensure the production of knot-free logs of sufficient quality for sawing. However the wounds that such practices involve may lead to fungal invasion which causes stem defects and degrade.

In order to assess the extent of fungal stem defect associated with pruning, a destructive survey was conducted in a 3-year-old *Acacia* hybrid plantation at Nghia Trung, Binh Phuoc province, 18 months after experimental thinning and pruning treatments. A total of 177 *Acacia* hybrid trees were felled for discoloration and decay assessment. Below 1.5 m tree height, the incidence of discoloration and decay in the pruned and thinned treatments was significantly higher than in the unpruned and unthinned treatments, respectively. The percentage of decay area was significantly greater in pruned than in unpruned trees and it was similar between thinning treatments. There was a significant pruning and thinning interaction for percentage of healthy wood and discoloration area but not for the discoloration and decay rating and the percentage of decay area. Above 1.5 m, all wood quality variables were unaffected by thinning and thinning x pruning interaction; discoloration and decay rating, and percentages of discoloration and decay areas were significantly greater (and consequently decreased the percentage healthy area) in pruned than unpruned trees.

Having established that there were significantly higher levels of discoloration and decay associated with pruned and thinned treatments compared to unpruned and unthinned treatments, the question is whether, as a tree grows, fungal decay and discolouration established at an early stage also increase in volume. It was decided to investigate the type of fungi associated with discoloration and decay as their identity may be indicative of the threat posed to solid wood production. Such fungi were examined in two *Acacia* hybrid plantations in southern Vietnam (Nghia Trung and Phan Truong Hai) by isolation into culture and direct sequencing from wood. A total of 231 fungal isolates were obtained from discoloured wood samples harvested from Nghia Trung and Phan Truong Hai. The DNA-based identification of isolates showed a wide diversity of fungal groups with three times as many ascomycetes as basidiomycetes. Approximately four years after the sampling for isolation and at harvest age, discoloured and decayed wood samples were taken from the same *Acacia* hybrid plantation at Phan Truong Hai. DNA was extracted directly from discoloured and decayed wood samples and associated fungi identified by 454 sequencing of fungal PCR products. Again there was a predominance of ascomycete fungi associated with discoloured and decayed wood with 68 fungi identified as Ascomycota and 41 as Basidiomycota and a low incidence of wood rotting basidiomycetes. Different silvicultural regimes did not influence the fungal communities associated with discoloured and decayed *Acacia* wood and there was no significant difference between fungal communities in discoloured wood compared to decayed wood. The low prevalence of wood decay basidiomycetes indicates that the risk of fungal stem defects at harvest is not as significant as previously considered.

Observations of the levels of discolouration and decay from Phan Truong Hai in the trees sampled at harvest support this hypothesis (data not shown in thesis).

Greater incidence and severity of vascular wilt diseases is commonly associated with mechanical wounding. *Ceratocystis manginecans*, a canker and wilt pathogen, was identified from wood samples at Nghia Trung and Phan Truong Hai. In SE Asia, the productivity of *Acacia* plantations is being severely threatened by this aggressive pathogen. Apart from growing a less susceptible plantation species, there are few opportunities in *Acacia* for the management of this disease apart from the use of tolerant clones. *Acacia* is typically singled at 6 months to ensure a single stem and, as previously mentioned, must be pruned and thinned for solid wood production. In addition, *Acacia* plantations are subject to wounding by boring insects and browsing mammals. A pot trial was established in Binh Duong province, southern Vietnam to screen for the host response of nine *Acacia* genotypes (six *Acacia* hybrid clones, two *A. auriculiformis* clones and mixed provenance seedlings of *A. mangium*) to artificial inoculation with three isolates of *C. manginecans*. Lesion lengths as measured on the inner bark indicated that the two *A. auriculiformis* clones were relatively more tolerant to *C. manginecans* than the *A. mangium* genotype. In contrast, the lesion lengths of all six *Acacia* hybrid clones fell between the *A. auriculiformis* and *A. mangium* genotypes. The study suggests that it is possible to select amongst *Acacia* hybrid clones for tolerance to *C. manginecans.* However the main focus of the study was to explore the chemical basis to tolerance and the potential for using host chemical response to early infection as a rapid bioassay for tolerance selection. Chemical analysis of crude sapwood extracts sampled from the lesion provided some

evidence that induced phenolic compounds, particularly tetrahydroxyflavanone, and condensed tannins may have a defensive role in the *Acacia* - *C. manginecans* pathosystem. However, these results were not consistent across individual *Acacia* hybrid clones and *A. mangium* genotypes and therefore not reliably indicative of the host susceptibility.

In summary, the research in this thesis investigated two major biotic threats to the productivity of *Acacia* hybrid especially when grown for solid wood products. From the *Acacia* hybrid plantation investigated at Nghia Trung there appeared to be significant levels of discolouration and decay associated with early pruning and thinning operations. The fungi associated with this type of stem defect are however very diverse and, even at harvest age, do not comprise those wood decay basidiomycetes commonly associated with heart rot. *Ceratocystis manginecans* is the most serious threat to productivity but there appears to be differential levels of tolerance across *Acacia* species and hybrids that can be exploited, although no clear chemical host response indicative of tolerance was demonstrated.

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Table of contents

List of Tables

List of Figures

Abbreviation

AA	*Acacia auriculiformis*
ACIAR	Australian Centre for International Agricultural Research
AH	*Acacia* hybrid
AM	*Acacia mangium*
ANOVA	Analysis of Variance
Cp	Compound
*d*b	Diameter of branches or pruning stubs
dbh	Diameter at breast height
*d*l	Log diameter
DNA	Deoxyribonucleic acid
HPLC	High performance liquid chromatography
INSDs	International Nucleotide Sequence Databases

ITS	Internal transcribed spacer
MARD	Ministry of Agricultural and Rural Development
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
NT	Nghia Trung
OB	Over bark
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PTH	Phan Truong Hai
RNA	Ribonucleic acid
SE Asia	South-East Asia
Sig.	Significance
UB	Under bark

UPLC	Ultra performance liquid chromatography
WAPDA	Waters acquity photo diode array

Chapter 1
Introduction

More than 2.6 million hectares of *Acacia* plantations have been established across South-East Asia (Harwood & Nambiar 2014b). Vietnam has more than 1.3 million ha of *Acacia* plantations that play very important social and economic roles, especially in the reduction of poverty in Vietnam (Phat 2011). *Acacia* hybrid (*A. mangium* x *A. auriculiformis*) is the most widespread plantation species with a total of approximately 400,000 ha (Harwood & Nambiar 2014b). Various clones of *Acacia* hybrid are planted to supply the domestic demand for pulpwood, wood chips and sawn timber (Bueren 2004; Nambiar et al. 2015).

All indications are that these non-native *Acacia* species established as plantations will be challenged by increasing numbers of disease and pest problems, this being a worldwide trend (Harwood & Nambiar 2014b). There have been several significant problems in SE Asia reported in *Acacia* since its inception in the 1970s and 1980s as a hardwood plantation tree (Old et al. 2000; Bennett 2011). These include stem cankers caused by fungi such as *Erythricium salmonicolor* (Berk. & Broome) Burds. (syn. *Corticium salmonicolor* - causal agent of pink disease), *Phomopsis* sp. and *Nectria* sp. (Old et al. 1996; Old et al. 2000) and heart rots (Ito & Nanis 1997; Yamamoto et al. 1998; Lee 2003; Nghia 2010, 2015).

In a tree the wood (xylem) is protected from microbial attack by the secondary plant surface (periderm and rhytidome), a barrier that prevents the entry of most potential

pathogens, and constitutive induced defence mechanisms in the bark (cortex and phloem) (Pearce 1996). A few pathogens are able to penetrate these outer tissues directly, but most xylem pathogens gain entry through wounds (such as those associated with singling, pruning and thinning in *Acacia*, see below) that expose this tissue and make it more vulnerable to attack. Insect borers prevalent in SE Asia (Old et al. 1996; Thu et al. 2010; Thu 2016a) can provide infection courts for fungi to enter the wood (xylem).

There are four broad groups of xylem fungal pathogens: wood decaying fungi, canker rots, endophytes and latent infections and vascular wilts (Pearce 1996). Wood decay fungi are the most studied group. Rayner (1996) and Rayner & Boddy (1988) recognized that these fungi have five distinctive strategies; heart rot, active pathogenesis, specialized opportunism, desiccation tolerance and unspecialized opportunism.

Active pathogens can colonize healthy sapwood, following direct penetration (e.g. *Ganoderma*) or infection through wounds whereas opportunist fungi are less aggressive, colonizing only functionally compromised sapwood, for example that associated with a major wound (Pearce 1996). Many heart rot fungi are opportunists, grow and cause decay within the heartwood, which is generally devoid of living cells and hence lacks the capacity for active response to infection. The pathogenicity of opportunist fungi is variable. Some fungi can attack bark and xylem tissues simultaneously (Blanchette 1982) and cause canker rots. Endophytic fungi isolated from normal wood (Boddy & Griffith 1989; Oses et al. 2008; Martin et al. 2015) can

develop under favourable environmental conditions and give rise to decay lesions. In vascular wilts the xylem is colonised (Yadeta & Thomma 2013), its structure is disrupted and the water relations most often irrevocably damaged (Park et al. 2013; Yadeta & Thomma 2013).

Thinning and pruning are common silvicultural techniques in temperate plantation management for solid wood production to produce knot-free wood (Ikonen et al. 2009; Hein et al. 2014; Moreno-Fernandez et al. 2014), and to increase log quality (Gerrand et al. 1997), saw-log volume (Gerrand et al. 1997; Medhurst et al. 2011) and saw-log value (Callister & Wiseman 2013). Pruning and thinning are also required for *Acacia* plantations in tropical environments if trees are being managed for solid wood (Beadle 2006; Trang et al. 2017). As *Acacia* trees have a tendency to produce multiple leaders from the base, singling is also carried out at 4-6 months after planting (Beadle et al. 2013a; Huong et al. 2016). Fertiliser is commonly applied at planting time or after establishment to increase the growth rate of *Acacia* species (Trieu 2007; Bon & Harwood 2016; Huong et al. 2016). A high rate of fertiliser application when planting *Acacia* can significantly increase the proportion of trees that require singling and form pruning (Bon & Harwood 2016).

The wounding associated with thinning and pruning in both temperate and tropical plantations is often reported to increase the risk of stem defect from wood decay fungi (Pinkard et al. 2004; Beadle et al. 2008; Trang et al. 2017) and disease from vascular wilts (Tarigan et al. 2011b; Jeger et al. 2016). The proportion of the *Acacia* hybrid estate being managed for solid wood, mainly for furniture is increasing (Kha

et al. 2012; Nambiar et al. 2015). Since *Acacia* trees in plantations grown for solid wood require singling, pruning and thinning, the risk of fungal invasion and associated loss in productivity is greater than in pulpwood plantations.

Up to the first report of *Ceratocystis manginecans* M. van Wyk, Al-Adawi & M.J. Wingf. in *A. mangium* Willd. (Tarigan et al. 2011a) one of the most publicised disease problems with *A. mangium* was wound associated heart rot (Lee & Maziah 1993; Lee et al. 1996; Ito & Nanis 1997; Mohammed et al. 2006). Partly as a result of the heart rot problem, growers stopped growing *A. mangium* on the Malaysian Peninsula (Lee 2004). Up to the time that this thesis was started there was little information about the incidence and severity of heart rot problems in *Acacia* hybrid. There was, however, information about the factors that may increase the incidence and severity of wood decay fungi.

There are clear opportunities to refine silvicultural management strategies in Vietnam to minimise the risk posed by wood decay fungi in *Acacia* hybrid grown for solid wood. Site differences in decay incidence in Tasmanian *Eucalyptus nitens* have been attributed mainly to branch size and status, attributes that were clearly related to local climate and nutrition (Mohammed et al. 2000). Stem decay incidence and severity was positively correlated with increasing diameter of pruned green branches, particular those with a diameter > 2 cm. Managing stocking densities to reduce the incidence of large branches is recommended in both temperate (Neilsen & Gerrand 1999) and tropical regimes (Beadle et al. 2015). Selection of desirable genetic traits may be a possible management tool to reduce decay incidence and spread. Family

and progeny trials have shown that the incidence of heart and wound rot is under moderately strong genetic control in, for example, *E. nitens* [heritabilities 0.27 to 0.4 and 0.6, respectively (Wardlaw et al. 2003)] and easily assessed during routine sampling for wood properties. *Acacia auriculiformis* A.Cunn. ex Benth. appears less susceptible to heart rot than *A. mangium* (Ito 2002) and greater resistance may be associated with the phenolic profile of heartwood in *A. auriculiformis* (Barry et al. 2005b; Mihara et al. 2005; Barry et al. 2006).

The vascular wilt pathogen, *C. manginecans* is causing large scale mortalities of *Acacia* plantations, especially to *A. mangium* in Indonesia, Vietnam and Malaysia (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015). By 2015 this *Ceratocystis* wilt and canker pathogen was affecting approximately 2000 ha of *Acacia* plantations across Vietnam (Plant Protection Department - MARD 2015). A recent study estimated that the incidence of this disease on *A. auriculiformis*, *A. mangium* and *Acacia* hybrid plantations (*Acacia mangium* x *A. auriculiformis*) ranged from 7.1-12.5%, 9.2-18.4% and 10.2-18.2%, respectively (Thu et al. 2016).

As for wood decay fungi, *C. manginecans* enters trees via wounds created by pruning and thinning activities (Tarigan et al. 2011b) although this may not be the only pathway as *Ceratocystis* causing tree diseases can be disseminated by root grafting (Bruhn et al. 1991; Wilson & Lester 2002) or insect associates (van Wyk et al. 2007; Jeger et al. 2016) that either vector the pathogen or generate frass (Jeger et al. 2016) containing fungal propagules. It has been shown in Indonesia that careful singling versus rough singling plus fungicide application on pruning wounds can reduce the

incidence of *Ceratocystis* up to more than 30% (Tarigan et al. 2012). However the application of fungicide is not feasible in extensive plantations such as in Indonesia or Malaysia (Lee 2004). It could be in smaller scale plantations of Vietnam although the use of fungicide would be an additional cost to the smallholder farmer (Griffin et al. 2011; Blyth & Hoang 2013; Beadle et al. 2015). In addition wounding may be inflicted by less easily controlled mechanisms such as by mammal browsing, including by macaques and squirrels (Brawner et al. 2015; Siregar pers. comm.). The optimal solution to combatting *C. manginecans* would be to find tolerance (the trees that have capacity to develop and grow after the infection of pathogen) to the pathogen. Tolerance or resistance to vascular wilts has been demonstrated in other wood tree species; commercial mango stocks in Brazil have proven moderately resistant to *C. fimbriata* Ellis & Halst. (Arriel et al. 2016); certain clones of *Eucalyptus pellita* are resistant to *C. fimbriata* (Guimaraes et al. 2010b; Rosado et al. 2016); elms have been bred to be resistant to two invasive vascular wilt fungi, *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier (Pecori et al. 2017).

Due to the severity of the damage caused by *C. manginecans* there is an urgent need for rapid and reliable resistance screening, carried out on a large scale to select for tolerant germplasm that can be tested by natural inoculation in the field. There have been a number of *Acacia* screening trials established in Indonesia, Malaysia and Vietnam (Tarigan et al. 2011a; Thu et al. 2012; Chen et al. 2013; Brawner et al. 2015; Tarigan et al. 2016). Different rapid resistance screening methods have been trialled e.g. the artificial inoculation of potted seedlings (Tarigan et al. 2011a; Trang

et al. 2018), the inoculation of branch segments with waxed ends or the injection of acacia phyllodes with a spore suspension (Aswardi Nasution, Pham Quang Thu, pers. com.). An alternative approach to resistance selection could be the development of biochemical markers that indicate, at an early stage of infection, whether the host will be tolerant or susceptible. The potential of chemical compounds to act as markers of resistance or susceptibility has been demonstrated for other woody host pathosystems (Ockels et al. 2007; Woodward et al. 2007; McPherson et al. 2014), though reports of operational use of these markers were not found.

The identity of fungi associated with decay and discolouration at an early stage after invasion of a wound may be indicative of the potential for fungal stem degrade and loss of productivity at the end of rotation. Some basidiomycete fungi are known wood decay pathogens and/or heart rot agents in *Acacia* such as *Phellinus noxius* (Corner) G. Cunn. (Lee et al. 1988), *Ganoderma applanatum* (Pers.) Pat. (Bakshi 1976), *Rigidoporus hypobrunneus* (Petch) Corner and *Tinctoporellus epimiltinus* (Berk. & Broome) Rayvarden (Lee & Maziah 1993; Lee & Noraini Sikin 1999). Ascomycete canker fungi are also associated with stem damage and often tree mortalities (Old et al. 2000), for example, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and pink disease caused by *E. salmonicolor* which has been known to cause significant damage in *Acacia* plantations across south east Asia (Old et al. 1996; Hadi & Nuhamara 1997; Old et al. 2000).

The study of fungal communities such as those associated with stem defect in woody trees was held back by an inability to identify species in their vegetative states.

Although reproductive structures can be diagnostic, they are not ideal because they are produced infrequently in the field and may not discriminate among cryptic species (Williams & Viviani 2016). Culturing from wood samples and the identification of any cultures obtained is also fraught with issues; some fungi do not culture easily and even if cultures are obtained a lack of keys and accessible type specimens could make this approach difficult (Cao et al. 2012). When working in the tropics these issues are often compounded by a poorer understanding of mycological taxonomy where the majority of unidentified fungi reside (Berrin et al. 2012). However, the adoption and dissemination of DNA (deoxyribonucleic acid)-and ribonucleic acid (RNA)-based molecular tools has greatly reduced the barriers to sampling and identifying fungi from vegetative material. There are a number of excellent reviews that have examined the current array of molecular techniques available to those interested in working with fungi (Crous et al. 2015; Dulla et al. 2016; Hibbett et al. 2016). Currently, most molecular diagnostic tools are based on the polymerase chain reaction (PCR) technology and sequence-based identification of fungi, namely, DNA barcoding using the ITS (internal transcribed spacer) region and DNA taxonomy using one or multiple genes in sequence alignments and employing tree-building tools to estimate phylogenetic relationships. In DNA barcoding, the user compares an unknown sequence against a sequence database (Schoch et al. 2012; Raja et al. 2017). The development of techniques such as next generation sequencing (Collins et al. 2013; Buermans & den Dunnen 2014; Illumina 2016) could make the identification of fungal communities (Bullington & Larkin 2015; Sun et al. 2016) including those fungi associated with wood decay more rapid and informative. These high-throughput sequencing methods outperform earlier

approaches in terms of resolution and magnitude. These methods are currently taking over as the primary tool to assess fungal communities of plant-associated endophytes, pathogens, and mycorrhizal symbionts, as well as free-living saprotrophs (Metzker 2010; Dannemiller et al. 2014; Herr et al. 2015; Oono et al. 2015) although challenges still remain (Lindahl et al. 2013).

1.1 Thesis Aim

The overall aim of this study was to gain information that might allow silvicultural best practice and resistance screening to reduce potential losses caused by wood decay fungi and *C. manginecans* in *Acacia* hybrid plantations managed for sawlogs.

Thesis Hypotheses

The following hypotheses were tested:

(i) The risk of heart rot in pruned and thinned *Acacia* hybrid after pruning and thinning increases. This hypothesis was tested by quantifying the stem discoloration and decay following pruning and thinning an *Acacia* hybrid plantation.

(ii) Fungi associated with the early stages of discolouration and decay after pruning or thinning are mainly opportunistic and may not threaten the production of high quality wood. This hypothesis was tested by identifying the

fungal communities associated with discoloration and decay following pruning and thinning *Acacia* hybrid plantations.

(iii) The early biochemical host response of *Acacia* to infection by *Ceratocystis* can be used to develop biochemical indicators of tolerance or susceptibility to the pathogen. This hypothesis was tested by screening for host response to *C. manginecans* in different species of acacia and clones of *Acacia* hybrid.

1.2 Thesis structure

This thesis consists of six chapters including an introduction chapter, a literature review chapter, three experimental chapters and a general discussion chapter. The three experimental chapters have been written in paper formats for submision. In brief:

Chapter 1: Introduction

This chapter gives an introduction to *Acacia* hybrid plantations in Vietnam, their importance and silvicultural management for solid wood products. It outlines the disease problems in *Acacia* hybrid plantations associated with the wounding carried out during the pruning and thinning required for solid wood products. It gives context to the research approaches taken in this thesis to achieve the overall objective of reducing losses and to the hypotheses posed.

Chapter 2: Literature review

This chapter critically reviews relevent literature to detail the information required to carry out the research in this thesis, to discuss research outputs and make recommendations to achieve impact. The different sections discuss the significance of diseases in tropical *Acacia* plantations of SE Asia and Vietnam; discuss the silvicultural management such as thinning, pruning, fertiliser and defect associated with those techniques; discuss the heart rot, cankers and vascular wilts in hardwood plantations. These discussions include heart rot problems, fungi associated with discoloration, heart rot, stem cankers and vascular wilts and also the identification of fungi associated with heart rot, cankers and wilts; discuss the host responses to fungal pathogens including screening for host tolerence to fungal pathogens, condensed tannins and flavonoids; and discuss managing the risk of stem defects in solid wood production by silvicultural techniques and selection of resistanr and tolerant hosts.

Chapter 3: Quantifying stem discoloration and decay following pruning and thinning an *Acacia* hybrid plantation

This chapter presents the results of an experiment that quantified stem discoloration and decay from a pruning and thinning *Acacia* hybrid plantation at Nghia Trung, Binh Phuoc province, southern Vietnam.

The specific questions asked were:

- do pruned trees have a higher incidence and severity of discoloration and decay than unpruned trees?
- do trees in the thinned treatment have a higher incidence and severity of discoloration and decay than in the unthinned treatment?
- does a thinning and pruning interaction increase the incidence and severity of discoloration and decay?
- do pruned trees have a higher severity of discoloration and decay than unpruned trees, above 1.5 m tree height?

A part of this chapter was presented at an IUFRO conference held in Yogyakarta, Indonesia in October 2012:

Trang, T. T.; Beadle, C.; Mohammed, C. 2012. Heart rot in plantation *Acacia* hybrid in Vietnam. Proceeding of International Conference on the impacts of climate change to forest pests and disease in the tropoics and Working party conference IUFRO 7.02.07: Diseases and insects of tropical forest trees. 8th-10th October 2012, Yogyakarta, Indonesia, pp 45-49.

This entire chapter was subsequently published in Forest Pathology:

Trang, T. T.; Glen, M.; Eyles, A.; Ratkowsky, D.; Beadle, C. L.; Mohammed, C. L., 2017. Quantifying stem discoloration and decay following pruning and thinning an *Acacia* hybrid plantation. Forest Pathology, 47, e12312.

Chapter 4: Wood-rotting basidiomycetes are a minor component of the fungal community associated with discoloured and rotten of living *Acacia* hybrid trees grown for sawlogs in South Vietnam

This chapter presents the identification of fungal agents associated with stem defects in two *Acacia* hybrid plantations grown for sawlogs in Vietnam and considered the risk that the identified fungi might pose to the *Acacia* hybrid wood at the end of the rotation.

The specific questions asked were:

- are the fungi associated with rot the same as those associated with discolouration?
- do thinning and fertiliser treatments affect the fungal community associated with decay and discolouration of *Acacia* hybrids?
- do wood decay fungi pose a risk to stem defects of *Acacia* hybrids?

A part of this chapter was presented at an IUFRO - INAFOR joint international conference held in Yogyakarta, Indonesia in July 2017:

Trang, T. T.; Glen, M.; Beadle, C.; Eyles, A.; Ratkowsky, D. and Mohammed, C. 2017. Fungal agents associated with stem defects in plantations of *Acacia* hybrids grown for sawlogs in South Vietnam. IUFRO - INAFOR joint International

Conference 2017. Promoting sustainable resources from plantation for economic growth and community benefits. Yogyakarta, Indonesia 24th – 27th July, 2017, p 104.

The main results of this chapter were subsequently submitted to Forest Pathology and are under review:

Trang, T. T.; Glen, M.; Beadle, C.; Ratkowsky, D. and Mohammed, C.: Wood-rotting basidiomycetes are a minor component of fungal communities associated with *Acacia* hybrid trees grown for sawlogs in South Vietnam. Forest Pathology.

Chapter 5: Screening for host respones in *Acacia* to a canker and wilt pathogen, *Ceratocystis manginecans*

This research investigated the responses of nine *Acacia* genotypes to wounding and artificial inoculation with a canker and wilt fungal pathogen, *Ceratocystis manginecans*.

The specific questions asked were:

- are the host responses of nine *Acacia* plantation genotypes different in three isolates of a canker and wilt pathogen, *C. manginecans* in term of under bark lesion length?
- does the phenolic chemistry (i.e. condensed tannins, total phenolics as well eight selected individual phenolic compounds) induced locally in the

sapwood of all *Acacia* genotypes relate to host tolerance as indicated by lesion size caused by inoculation with three *C. manginecans* isolates?

The results of screening *Acacia* with *C. manginecans* were presented at an IUFRO - INAFOR joint international conference held in Yogyakarta, Indonesia in July 2017:

Trang, T. T.; Glen, M.; Beadle, C.; Eyles, A.; Ratkowsky, D. and Mohammed, C. 2017: Screening for host responses to a canker and wilt pathogen, *Ceratocystis manginecans*, in *Acacia* plantation genotypes. IUFRO - INAFOR joint International Conference 2017. Promoting sustainable resources from plantation for economic growth and community benefits. Yogyakarta, Indonesia 24th – 27th July, 2017, p 97.

The screening for host responses to a canker and wilt pathogen, *Ceratocystis manginecans* chapter has also been published in Forest Pathology:

Trang, T. T.; Glen, M.; Beadle, C.; Eyles, A.; Ratkowsky, D. and Mohammed, C. 2018: Screening for host responses to a canker and wilt pathogen, *Ceratocystis manginecans*. Forest Pathology, 48, e12390.

Chapter 6: Screening for host respones in *Acacia* to three white rot basidiomycete fungi

Initially, this chapter investigated the responses of nine *Acacia* genotypes to wounding and artificial inoculation with three white rot basidiomycete fungi.

The specific questions asked were:

- are the host responses of nine *Acacia* plantation genotypes different in three white rot basidiomycete fungi in term of under bark lesion length?
- does the phenolic chemistry (i.e. condensed tannins, total phenolics as well eight selected individual phenolic compounds) induced locally in the sapwood of all *Acacia* genotypes relate to host tolerance as indicated by lesion size caused by inoculation with three white rot basidiomycete fungi?

However, due to the attack of squirrels, five *Acacia* genotypes had been destroyed and therefore the experiment was undertaken with four *Acacia* genotypes.

Chapter 7: General discussion

This chapter presents a synthesis and discussion of the results presented in the 3 experimental chapters. It recommends appropriate management strategies that can be immediately adopted and approaches for future research to improve *Acacia* hybrid productivity by reducing losses caused by fungal agents.

Chapter 2
Literature review

2.1 *Acacia* plantations in South-East Asia

More than 2.6 million hectares have been planted with *Acacia* species in South-East Asian countries (Harwood & Nambiar 2014b). *Acacia* plantations are increasing in SE Asia and Vietnam to meet multiple needs; the domestic demand for wood products, increased national export revenue, support for the rural economy and poverty reduction (Nambiar et al. 2015). Vietnamese plantations (46% of the total plantation area) are predominantly grown by private smallholders (Nambiar et al. 2015). Other growers include forest management boards (17%), state companies (15%),community committees (12%), private companies (4%) and other minor owners (Kien et al. 2014).

Acacia species were introduced from their natural distribution in northern Australia and Papua New Guinea to southern Vietnam in the 1960s and to northern Vietnam in the early 1980s (Nambiar et al. 2015). Vietnam has about 1.3 million ha of *Acacia* plantations which include 600 000 ha of *A. mangium*, 90 000 ha of *A. auriculiformis* and over 400 000 ha of *Acacia* hybrid (Nambiar & Harwood 2014).

The best-performing pure species (*A. auriculiformis*, *A. crassicarpa* and *A. mangium*) and selected clones of the interspecific hybrid (a natural hybrid between *Acacia mangium* x *Acacia auriculiformis*) are currently the most widely planted *Acacia* genotypes in Vietnam (Kien et al. 2014). The popularity of *Acacia* hybrid has

resulted from the selection and rigorous testing of clones that demonstrate consistently high growth rates across a range of lowland site types in northern, central and southern Vietnam (Kha et al. 2012). Although *Acacia* hybrid plantations in Vietnam are grown to supply raw materials for pulp, chipboard and paper (Kha 2000), an increasingly significant proportion are being managed for sawn timber, mainly destined for furniture manufacture (Kha 2000; Harwood et al. 2006; Nambiar et al. 2015).

2.2 Significance of diseases in tropical *Acacia* plantations of SE Asia especially Vietnam

Australian acacias and eucalypts planted as non-natives in SE Asia and other parts of the world are increasingly threatened by pests and pathogens as plantation estates age, especially in SE Asia (Lee 2003; Wingfield et al. 2011; Harwood & Nambiar 2014b). These include accidental introductions from the same areas of origin as the trees, as well as 'new encounter' pests and pathogens that are undergoing host shifts to infect the non-native trees (Burgess et al. 2007). Pests and pathogens do not recognise national borders and shared host species in SE Asia implies shared pest and pathogens (Old 2002).

The occurrence of both root and heart rots in tropical acacias in SE Asia has received considerable attention (Lee 2003; Lee 2004). Species of *Ganoderma* and *Amauroderma* are associated, either as primary or secondary pathogens, with root rot in *Acacia* plantations in Indonesia and Malaysia (Glen et al. 2009; Coetzee et al.

2011; Puspitasari et al. 2012). In Sumatra, Indonesia, root rot caused by *Ganoderma philippii* (Bres. & Henn. ex Sacc.) Bres. was sufficiently damaging to drive the planting of eucalypt species in preference to *A. mangium* as eucalypts appears less susceptible to *G. philippii* (Mohammed et al. 2014). *Ganoderma philippii* has not been recorded from *Acacia* plantations in Vietnam and significant damage from basidiomycete root pathogens is not apparent (Nghia 2015). However, significant areas of young *A. mangium* and *Acacia* hybrid plantations in northern Vietnam (Tuyen Quang, Phu Tho, Yen Bai, Lao Cai and Lai Chau) have been killed by root rot caused by *Pythium vexans* (syn. *Phytopythium vexans* (de Bary) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque) and species of *Phytophthora* and *Phytopythium* (Nghia 2015).

Acacia species including *A. mangium* and *A. auriculiformis* in Indonesia and Malaysia are affected by heart rot (Zakaria et al. 1994; Lee 1996; Barry et al. 2004; Rimbawanto 2006) but Ito & Nanis (1994) found that *A. mangium* was more susceptible to heart rot than *A. auriculiformis*. Incidence differs between regions, ranging from low to high depending on the combination of climate, plantation management and age (Arisman & Hardiyanto 2006). If the incidence and severity of heart rot is high it can reduce timber volume and quality in trees grown for solid wood production (Potter et al. 2006). In Sabah the average heart-rot incidence in *A. mangium* plantations of 6-9 years was 35.5% with up to 18.1% of merchantable volume affected by decay (Mahmud et al. 1993). Heart-rot incidence of *Acacia mangium* in Peninsular Malaysia was reported as ranging from 57-98% in 2 and 8 year-old trees with a loss of 17% volume of the merchantable timber of *Acacia*

mangium (Zakaria et al. 1994). Due to this heart rot problem, the Malaysian Government had to review its policy on the further development of *A. mangium* plantations in Peninsular Malaysia (Lee et al. 1996). Heart rot, however, causes little problem for the pulpwood industry (Gales 2002) as the white-rot fungi causing heart rot preferentially remove lignin which must be removed by chemical treatment for pulp production.

Heart rot has been reported in *Acacia* plantations in Vietnam, especially when *Acacia* plantations are grown for sawlogs with a rotation of 10-15 years (Thu 2009; Nghia 2015) but the incidence is not as high as reported in Indonesia and Malaysia. The heart rot incidence in a 12-year-old *A. mangium* plantation in Song May, Dong Nai province has been given as 14.2% and in a 15-year-old *A. mangium* plantation in Ham Yen, Tuyen Quang province as 23.6% (Thu 2009). The main heart rot causal agents cited are *Ganoderma tropicum* (Jungh.) Bres. and *G. australe* (Fr.) Pat. but there has not been any molecular verification (Nghia 2015).

The best known and most damaging canker disease associated with tropical *Acacia* plantations is pink disease (Old et al. 2000). This disease is caused by the basidiomycete fungus, *Erythricium salmonicolor* (syn. *Corticium salmonicolor*), which has a very wide host range, including rubber (Hilton 1958), eucalypts (Sharma et al. 1988), *A. mangium* (Hadi & Nuhamara 1997), *A. crassicarpa* and *A. aulacocarpa* (Hadi & Nuhamara 1997) and *A. auriculiformis* (Sharma & Florence 1996). The pathogen can invade the tree through healthy bark tissue and is favoured by high humidity and areas where many susceptible plantation species such as

tropical fruit trees, cocoa, coffee and other crops are grown (Old et al. 2000). Pink disease is one of the most important fungal diseases in *Acacia* plantations in Vietnam (Nghia 2015; Thu 2016b) especially in southern Vietnam e.g. 158 ha of *A. mangium* in Ha The, Lam Dong province were impacted by this disease in 2002 (Nghia 2015). Its main impact is loss of stem form through the loss of the leading shoot (Old et al. 2000). Certain Vietnamese clones of *Acacia* hybrid are highly susceptible, others resistant (Nghia 2015).

While the most significant diseases of *Acacia* plantations in tropical areas of SE Asia have been historically considered to be stem cankers, heart rot, butt and root rots as described above (Old et al. 2000; Thu et al. 2010), over the last decade a new *Acacia* disease has rapidly risen to devastating prominence. This is a vascular wilt and stem canker caused by *Ceratocystis manginecans*, causing large scale mortalities (thousands of hectares), especially in *A. mangium*, in Indonesia, Malaysia and Vietnam (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015; Fourie et al. 2016). The trend of replacing acacia with eucalypts was accelerated with the advent of Ceratocystis wilt and stem canker disease in *Acacia*, not just in Indonesia but also in Malaysia (Harwood & Nambiar 2014b; Mohammed, pers. comm).

Ceratocystis wilt and stem canker has been found to damage all of the main *Acacia* species planted in Vietnam i.e. *A. auriculiformis, A. mangium* and *Acacia* hybrid (Nghia 2015; Thu et al. 2016). By 2015 this *Ceratocystis* wilt and canker pathogen was affecting approximately 2000 ha of *Acacia* plantations across Vietnam (Plant Protection Department - MARD 2015). A recent study estimated that the incidence

of this disease on *A. auriculiformis*, *A. mangium* and *Acacia* hybrid plantations ranged from 7.1-12.5%, 9.2-18.4% and 10.2-18.2%, respectively (Thu et al. 2016).

In summary, if plantation forest productivity is to be sustained, the implementation of effective pest and disease management is an immediate priority for SE Asia. This message is highlighted by the recent statement by Harwood & Nambiar (2014a) that due to tree mortality, growth rates of *A. mangium* in Sumatra in those areas impacted by fungal diseases (*Ganoderma* and *Ceratocystis*) have been reduced to less than 15 m^3/ha/yr, while non-impacted areas have growth rates in the range of 22 to 35 m^3/ha/yr.

2.3 Silvicultural management in *Acacia* and wounding associated stem defects

Silvicultural techniques that utilise pruning and thinning are of crucial importance in growing plantations for solid wood to maximise their productivity and quality (Gerrand et al. 1997; Beadle 2006) and *Acacia* plantations grown for solid wood are no exception (Beadle et al. 2013b; Beadle et al. 2013a). Both of these techniques however may be associated with the development of fungal associated stem defects in temperate and tropical hardwood plantations (Pinkard et al. 2004; Beadle et al. 2008; Trang et al. 2017) and disease from vascular wilts (Tarigan et al. 2011b; Jeger et al. 2016). Singling is another silvicultural operation resulting in wounding and often done in commercial *Acacia* plantations to improve tree form, restore apical

dominance, increase tree strength and reduce the number of co-leader shoots so that optimum tree growth can be achieved (Tarigan et al. 2012; Beadle et al. 2013b).

2.3.1 Thinning

Thinning is a silvicultural treatment applied to overstocked, even-aged stands to release potential sawlogs from competition (Forestry Tasmania 2001). Thinning enables selection of trees with the best form (Beadle et al. 2007; Beadle et al. 2013a) and increases the growth rates of individual trees (Wood et al. 2009; Forrester et al. 2010), saw-log volume (Gerrand et al. 1997; Wardlaw 2003; Medhurst et al. 2011) and saw-log value (Beadle et al. 2013a).

Historically, plantation systems grown for sawlog production usually require at least one thinning to manage stand stocking and increase the diameter of retained trees (Beadle et al. 2013b). Early thinning has been demonstrated to improve future log quality and value without compromising stand stability and long-term financial viability (Cameron 2001) and allows the best trees in the stand to take advantage of any improved growing condition such as light, moisture, space and nutrients to maintain active growth and, therefore their sizes increase more rapidly (Hardiyanto 2006).

Optimum thinning regimes have been tested and developed for a range of fast-growing plantation species including various temperate and tropical *Eucalyptus* spp. (Medhurst et al. 2001; Smith & Brennan 2006; Cassidy et al. 2012). Studies

demonstrate that thinning regimes may require between 1 to 5 thinning events at both early and later ages depending on sawlog specifications at harvest (Medhurst et al. 2001; Kanninen et al. 2004; Glencross et al. 2014). In contrast, there is little published research on thinning for fast-growing tropical *Acacia* spp. (Beadle et al. 2013a; Huong et al. 2016).

In a comparison of thinning treatments of *Acacia* hybrid in southern Vietnam, Huong et al. (2016) concluded that a single thinning from 1111 trees ha^{-1} to 600 trees ha^{-1} at age 2 years or double thinning to 833 trees ha^{-1} then 600 trees ha^{-1} at age 2 and 3 years, respectively, would produce the highest diameter sawlogs. Early thinning of a 2.5-year-old *Acacia* hybrid plantation from 1000 to 600, 450 and 300 trees ha^{-1} was shown to rapidly increase sawlog values (defined as log DBH >15 cm in small-end diameter) in central Vietnam (Beadle et al. 2013a). While the findings from these studies suggest that *Acacia* plantations with relatively low initial stockings can be managed to produce sawlogs, initial stockings in Vietnam are more commonly at least 1600 tree ha^{-1} and often higher (Beadle et al. 2015). How to manage these plantations for sawlogs remains unclear.

Thinning can increase the risk of stem defects (Han & Kellogg 2000; Barry 2002). Hunt & Krueger (1962) found that 61 and 23% of the wounds caused by thinning plantations of *Tsuga heterophylla* (Raf.) Sarg. and *Pseudotsuga menziesii* (Mirb.) Franco, respectively, were associated with decay. In 19- to 20- year- old thinned stands of *Eucalyptus regnans* F. Muell., 8.7–15.7 % of wounds were associated with discoloration and decay (White & Kile 1991). Given the relatively high number of

thinning operations required (and carried out manually in often densely stocked plantations) there are clear opportunities for stem wounding and potential stem decay infections in *Acacia* plantations. Until the research of this thesis there were no data pertaining to the incidence and severity of thinning associated decay in *Acacia*. Trang et al. (2017) found that thinning increased the incidence of stem decay and the severity of discoloration but not the severity of decay. An interaction between pruning and thinning did not influence the incidence of stem decay but did increase the severity of discoloration in thinning treatments.

2.3.2 Pruning

Fast growing plantation trees such as acacias and eucalypts require pruning because they do not naturally shed dead lower branches. Dead branches often persist for a long time and are associated with a deterioration in wood quality (Wardlaw et al. 2003). In *Acacia*, dead branches may also be associated with a high percentage of discolouration and decay (Ito & Nanis 1994). In Australian eucalypt plantations, pruning usually occurs while branches are still living (Montagu et al. 2003; Pinkard et al. 2004) and the same practice has been adopted for *Acacia* (Beadle 2006). Pruning restricts the development of loose knots and decreases the size of the knotty core. Trees therefore develop a straight stem and more valuable, knot-free trunks (Moreno-Fernandez et al. 2014) and the amount of clear wood produced by trees is maximised (Hardiyanto 2006). The extent to which trees can be pruned before their growth is significantly reduced is a function of species and site (Beadle et al. 2008). In general, the growth of slower-growing tree species is more affected by pruning

than that of faster-growing species such as acacias and eucalypts (Beadle et al. 2008). Removal of branches from 50% of the crown length of *E. nitens* had negligible impact on height or diameter growth provided pruning was done after canopy closure (Pinkard et al. (1998).

In *Acacia* three different styles of pruning have been trialled; form pruning, lift pruning and tip pruning. Form pruning removes a percentage of leaf area by removing large branches and those subtending a narrow angle with the stem up to a designated height while lift pruning removes a percentage of crown length from below (Beadle et al. 2007). Tip pruning removes a proportion of the length of branches and competing leaders with the aim of promoting dominance in the unpruned leader (Beadle et al. 2013b).

In addition to studying the impact of pruning on the quality and quantity of solid wood produced from *Acacia* by pruning, there has also been a parallel consideration that pruning wounds are potential infection courts for fungi. Beadle et al. (2007) investigated the effects of pruning on both stem form and the incidence of heart rot in an 18-month-old plantation of *A. mangium* in South Sumatra. The objectives were to assess whether pruning is associated with an increase in the incidence of heart rot and whether form pruning compared to lift pruning reduced the incidence of heart rot and improved stem form. No significant difference in diameter increment between the two pruning treatments was found but there was strong evidence that form pruning was associated with better form 18 months after treatment. Lift pruning reduced average branch size but did not improve stem straightness. The results

showed that form pruning is likely to have positive benefits on stem straightness and is likely to be effective to any selected pruning height but a subsequent lift pruning is still considered a requirement. No heart rot was detected in any treatment and it was concluded that, although wounds created from pruning and singling are assumed to have a large impact on the incidence of heart rot, this may not be an issue unless there is a sufficient source of fungal inoculum present in a favourable environment to invade the wounds. In contrast, the research in this thesis showed that pruned *Acacia* hybrid in southern Vietnam had a higher incidence and severity of discoloration and decay in the stem than unpruned trees (Trang et al. 2017). This result might be attributed to the climatic conditions prevailing at pruning and directly afterwards which were more humid and wet compared to those in Sumatra. These environmental conditions favour fungal sporulation and infection.

Beadle et al. (2013b) have developed pruning prescriptions for lift pruning and tip pruning for *Acacia* hybrid in Vietnam which also target the reduction of fungal infections. Lift pruning is recommended to be undertaken only in the dry season to minimise the risk of pathogen entry through pruning wounds adjacent to the stem. Tip pruning as a type of form pruning which removes approximately one-half of the length of competing leaders and large diameter branches is recommended. The advantages of tip pruning are: dominance of competing stems or leaders is removed but leaf area and therefore growth potential is retained and excision of unhardened stems/branches at the point where they join the retained stem is avoided, theoretically reducing the potential for disease entry.

2.3.3 Fertiliser

Fertiliser application is usually used to increase growth rates of trees in fast growing plantations (Forrester et al. 2013). In Vietnam, fertiliser is normally applied at planting time, six months and to up to two years after establishment to increase the growth rate of *Acacia* species (Trieu 2007; Bon & Harwood 2016; Huong et al. 2016).

Wiseman et al. (2006) found that decay infections from pruned stubs of *Eucalyptus nitens* in Tasmania were more common in trees that received additions of N and P fertiliser, hence improved tree nutrition increased the longevity and size of branches, thereby leading to an increased incidence of decay infection. Similarly high rates of fertiliser application at establishment of *Acacia* hybrid plantations has been suggested to increase the proportion of trees requiring singling and form pruning (Bon & Harwood 2016) which may lead to a greater risk of wood decay fungi infecting stems of trees.

2.4 Heart rot, cankers and vascular wilts in trees

2.4.1 Heart rot

Heart rot is decay in the central stem or heart wood but some fungi also attack sapwood and cause sapwood rot (Vasaitis 2013). When functional sapwood is killed by a decay fungus this is called a canker rot and the same fungus may simultaneously cause heart rot, sapwood rot and canker rot (Vasaitis 2013). Manion (1990)

overviewed the four different concepts of infection and colonisation of a tree by fungi; i) the 'Haddow-Etheridge' concept which describes latent establishment (Haddow 1938; Etheridge & Craig 1976), ii) the 'Hartig' concept of fungi that infect a tree via a wound that exposes heartwood (Hartig 1874), iii) the 'Shigo' concept of fungal succession after entry via sapwood wounds (Shigo 1966), iv) the 'Boddy-Rayner' concept explaining fungal infection patterns in terms of moisture content (Boddy & Rayner 1983). Vasaitis (2013) categorises the fungi that infect living trees as i) true heart rot fungi that gain entry via natural infection courts, ii) true heart rot fungi that infect via natural wounds such as fire scars or broken branches, iii) wound heart rot fungi that enter through dead sapwood and comply with the Shigo concept (Shigo 1966). While these categories may reflect the best knowledge available at the time, new insights into fungal lifestyles may be gained in the near future through advances in metagenomics (Zhao et al. 2015). Discoloration is defined as "an early stage of decay where decomposition remains absent" (Mahmud et al. 1993).

Apart from mechanical wounds associated with silvicultural management such as singling, pruning and thinning, infection courts for heart-rot fungi can be branch stubs and dead branches (Mahmud et al. 1993; Ito & Nanis 1994, 1997; Barry et al. 2004). Injuries caused by broken branches after self-pruning, singling and artificial pruning and stem cankers (Lee 2002) and cause decay inside living trees (Bougher & Tommerup 2002; Lee 2002).

There has been a lack of consistency in methods for heart rot assessment leading to development of different types of rating systems. Wardlaw & Neilsen (1999) used a

rating system that did not differentiate between decayed and discoloured wood of *E. nitens*. Wardlaw et al. (2003) quantified decay percentage as stem volume where severe decay was ≥5% and 2 to <5% was moderate in intensively managed 22- to 34-year-old plantations of *E. nitens*. Mahmud et al. (1993) developed a six–category rating system to evaluate discoloured and heart rotted wood of *A. mangium* whereas Barry et al. (2004) developed a five–category rating system to assess heart rot severity of *A. mangium*. Both of them were qualitative and based on rough visual estimations of discoloration and decay. These different approaches highlighted the need to develop a consistent rating system for quantifying the impacts of management practices on the development of discoloration and decay and their interrelatedness (Trang et al. 2017).

The many permutations of woody host species and lignicolous fungi can produce many kinds of wood rot but these are traditionally divided into three broad types, which may be termed brown rot, white rot or soft rot (Liers et al. 2011) (Table 1 in Schwarze 2007). White rot is caused by simultaneous degradation of lignin and cellulose by enzymes including laccase and tyrosinase (Padhiar et al. 2010; Liers et al. 2011) resulting in a soft, spongy or stringy, white or yellowish material. In brown rot, cellulose is degraded by enzymes and small molecules including hydrogen peroxide, but lignin degradation is not as pronounced; the wood turn brown, shrinks and may break into small cubical fragments (Rayner & Boddy 1988; Worrall et al. 1997). Soft rot fungi are predominately members of the Ascomycota and also secrete cellulases, but these attack different cell wall components (Table 2-1). Soft-rot fungi require a source of nitrogen to produce enzymes, are less competitive than brown or

white rotters, but are able to tolerate conditions that are too hot, too cold or too wet for the other types (Blanchette 2000).

Table 2-1: Fungal mechanisms of wood decay [from Blanchette 2000]

Decay type	Wood component utilised	Decay characteristics
White rot	All cell wall components, some species preferentially attack lignin	Progressive erosion of all cell wall layers. Middle lamella is degraded.
Brown rot	Carbohydrates, some lignin modification.	Diffuse depolymerisation of cellulose
Soft rot	Carbohydrates, some lignin modification.	Type 1 - cavities form in secondary wall Type 2 - progressive erosion of secondary walls but middle lamella is not degraded

More recently, genomic studies have highlighted the complexity of wood decay processes and reinforced the idea that the brown rot/white rot distinction in Basidiomycota does not represent a precise division as some species may exhibit intermediate behaviours (Floudas et al. 2015). Schilling et al. (2015) have proposed a measure to place wood decay basidiomycetes on a spectrum with white at one end and brown rot at the other. The high diversity of rot types reflects the nutritional

strategies of fungi and is not surprising given the large number of enzymes that can mediate wood degradation, e.g. 110 potential plant cell wall-degrading enzymes in the white-rot fungus *Obba rivulosa* (Berk. & M.A. Curtis) Miettinen & Rajchenb. (Marinovic et al. 2017). However, genomic prediction of enzyme activity may not correlate well with enzyme screening results (Eichlerová et al. 2015). While expression of some enzymes is constitutive, e.g. endoglucanase in the brown rot fungus *Fomitopsis palustris* (Berk. & M.A. Curtis) Gilb. & Ryvarden (Hong et al. 2017) or manganese peroxidase in *Obba rivulosa* (Marinovic et al. 2017), others may be inducible, e.g. in the brown rot fungus *Postia placenta* (Fr.) M.J. Larsen & Lombard, cellulases and oxidorectases are induced and repressed (respectively) by cellobiose (Zhang & Schilling 2017). This inducibility allows the fungus to change its gene expression profile as decay proceeds (Skyba et al. 2016). Different tree species vary in their cell wall composition and so expression of different genes may also be induced in wood from different tree species (Gaskell et al. 2016).

Heart rot fungi have been studied more intensively and over a longer period in temperate forests than in tropical forests (Appendix 2-1). Vasaitis (2013) provides a thorough review of heart rot agents in Northern Hemisphere temperate and boreal forest stands. Only three of the species listed in Appendix 2-1 are members of Ascomycota (bold text in Appendix 2-1), the rest are Basidiomycota with the majority of species belonging to Hymenochaetales or Polyporales. Other fungal families represented include the Agaricales and Boletales. The major representative of the Agaricales is *Armillaria* (Strophariceae), a well-known genus of plant pathogens that cause root and butt rots (Guillaumin et al. 1993) as well as heart rot

(Arhipova et al. 2012). Other Agaricales species include *Mycena galericulata* (Scop.) Gra (Mycenaceae), *Pholiota adiposa* (Batsch) P. Kumm. and *P. aurivella* (Batsch) P. Kumm. (Strophariaceae). The first and third were isolated from a low percentage of *Alnus glutinosa* trees with stem rot (Arhipova et al. 2012) and the second is reported from Western hemlock in Alaska (Hennon 1995).

Some fungal species that cause heart rot are widespread and occur on many host species e.g. *Fomitopsis pinicola* (Sw.) P. Karst. occurs in a wide variety of conifers and is distributed across the temperate and boreal regions of the northern hemisphere (Vasaitis 2013). Other species are more localised to a particular region or host species, e.g. *Cinereomyces lindbladii* (Berk.) Jŭlich is reported only from *Alnus glutinosa* in Latvia (Arhipova et al. 2012). The broad distribution of some species may be due to poorly resolved taxonomy as is suggested for *Laetiporus sulphureus* (Bull.) Murrill (Rogers et al. 1999), which has been implicated in heart rot of both conifers and hardwoods. Further studies have supported this argument for *L. sulphureus* (Banik et al. 2010). *Ganoderma* is a major genus of heart rot agents in the Polyporales (Ganodermataceae) in both tropical and temperate regions (Hong 1982; Guglielmo et al. 2007). *Phellinus* is the major genus of heart rot agent representative of Hymenochaetales (Hymenochaetaceae) and among the most frequently cited causal agents of heart rot (Lee et al. 1996; Guglielmo et al. 2007; Brazee 2015; Rajchenberg et al. 2015) in both temperate and tropical systems.

Although *Acacia* species have been widely planted in South-east Asia and the occurrence of heart rot has been noted (Old et al. 2000; Lee 2003; Lee 2004), the

causal fungi have not been well studied in this region. In Malaysia, a number of different fungi have been isolated from *A. mangium* stems with heart rot but identification of the fungal species has often been incomplete due to the lack of fruiting structures and molecular identification (Lee et al. 1988; Hashim et al. 1990; Lee & Maziah 1993; Lee & Noraini Sikin 1999). Seven main types of rot have been described from rotted *A. mangium* heartwood, with white fibrous rot the most often encountered type of rot. Fungal isolates obtained have been classed as Hymenomycetes isolates including *Rigidoporus hypobrunneus* (Petch) Corner, *Phellinus noxius* (Corner) G. Cunn., *Tinctoporellus epimiltinus* (Berk. & Broome) Rayvarden and *Oxyporus* cf. *latemarginatus*.

In Vietnamese *Acacia* plantations (*Acacia auriculiformis, A. mangium* and *Acacia* hybrid) heart rot has been attributed to species of *Ganoderma* and *Trametes*, *Phellinus pachyphloeus* (syn. *Inonotus pachyphloeus* (Pat.) T. Wagner & M. Fisch.) and *Pycnoporus coccineus* (Fr.) Bondartsev & Singer) (Nghia 2006, 2010, 2015; Thu 2016b). Recently, *Acacia* plantations, especially *Acacia* hybrid plantations in Vietnam are expanding rapidly to meet the needs of the wood processing industry as well as the pulp and paper industry (Bueren 2004; Nambiar et al. 2015). Managing the risk of heart rot will assist plantation owners to maximise profits from their plantations. An understanding of the fungal agents responsible for causing heart rot is fundamental to managing this risk.

2.4.2 Stem cankers

Cankers can be classified as belonging to four types; saprophytic, annual, perennial or diffuse (Manion 1990). The fungi that are associated with these cankers have different levels of pathogenicity e.g. saprophytic cankers are caused by fungi that can only invade compromised tissue. Annual and perennial cankers although associated with greater fungal pathogenicity, according the Manion 1990, are still caused by opportunistic fungi invading open wounds, dead branches and branch stubs. Fungi are restricted to bark and xylem tissues that are killed by fungal toxins or enzymes. Target shaped perennial cankers are formed when the host responds by forming callus tissue, and then the fungus overcomes the resistant response and overgrows the callus tissue. Annual layers of callus are formed, giving the appearance of a target. Diffuse cankers are caused by the most pathogenic fungi and destroy the cambium of the stems as lesions develop along the stems, the fungi provoke minimal response by the host.

Cankers can be incited by many different types of fungi from Ascomycota, Oomycota or Basidiomycota. A recent example of a devastating canker disease caused by an oomycete fungus is sudden oak death (SOD) in natural forests in California and Oregon (Garbelotto & Hayden 2012), as well as being present in Europe (Marcais et al. 2004; Grunwald et al. 2008). The causal agent *Phytophthora ramorum* Werres, De Cock & Man in 't Veld is aerially dispersed (Grunwald et al. 2008; Harris & Webber 2016). Bole or trunk cankers form on tanoaks and some oaks

while most shrubs and non-woody plants show leaf spots which may be accompanied by shoot dieback. Trunk cankers are the most damaging, and often lead to death.

Quambalaria are basidiomycete species implicated in canker and shoot blight diseases of *Corymbia* species e.g. *Quambalaria coyrecup* Paap on *Corymbia ficifolia* in Western Australia (Yulia et al. 2014). *Quambalaria eucalypti* (M.J. Wingf., Crous & W.J. Swart) J.A. Simpson has been detected on *E. grandis* in Australia (Carnegie 2007), South Africa (Wingfield et al. 1993; Roux et al. 2006) but is only significantly damaging in Europe or various sub-tropical or tropical countries of the southern hemisphere (Ferreira et al. 2008) where eucalypts are exotic (Bragança et al. 2016). Many basidiomycetes are canker rots that are able to attack the bark and xylem tissues simultaneously e.g. *Inonotus obliquus* (Fr.) Pilát on *Betula* spp. and *Phellinus pini* (syn. *Porodaedalea pini* (Brot.) Murrill) on *Abies* spp. (Pearce 1996).

Canker diseases caused by ascomycete fungi are among the most damaging forest diseases worldwide and may be highly invasive e.g.

- *Castanea dentata* (Marsh.) Borkh. was a dominant forest tree species extending over 800,000 ha in north America until the accidental introduction from Asia of *Cryphonectria parasitica* (Murrill) M.E. Barr in the early 1900s (Jacobs et al. 2013). Within 50 years, this pathogen had rapidly annihilated *C. dentata* throughout its range, with restoration efforts now focussing on establishment of resistant populations (Clark et al. 2014). Some of the most significant and globally occurring stem canker pathogens of tropical and sub-

tropical plantation eucalypts belong to the Cryphonectriaceae. Typical symptoms of these pathogens include diffuse, sunken or deformed cankers on roots, trunks and branches which extend longitudinally and laterally (Wingfield 2003; Vermeulen et al. 2013) with bleeding kino (Vermeulen et al. 2013) e.g. *Chrysoporthe cubensis* (Bruner) Gryzenh. & M.J. Wingf. (syn. *Cryphonectria cubensis*) and *C. deuterocubensis* Gryzenh. & M.J. Wingf. (Van der Merwe et al. 2010) and *C. austroafricana* Gryzenh. & M.J. Wingf. (Heath et al. 2006; Nakabonge et al. 2006).

- Pine pitch canker, caused by *Fusarium circinatum* Nirenberg & O'Donnell, native to Central America, causes significant economic losses around the world in many pine species (Wingfield et al. 2008; Bezos et al. 2017).

- *Botryosphaeria dothidea* (Moug.) Ces. & De Not., an ascomycete, is a common canker fungus with a worldwide distribution and associated with many hosts including plantation eucalypts and acacias (Marsberg et al. 2017). Infections typically become obvious only under conditions of host stress, when disease symptoms develop. The prolonged latent infection or endophytic phase is of particular importance and the fungus can easily pass undetected by quarantine systems in plants or plant parts.

The best known canker disease associated with tropical *Acacia* plantations (see Section 2.2), including *Acacia auriculiformis, A. crassicarpa* and *A. mangium* in many SE Asian countries (Chin 1990; Lee 1993; Hadi & Nuhamara 1997; Old et al.

2000) is pink disease caused by *E. salmonicolor*. In addition to *E. salmonicolor,* a diverse range of fungi have been isolated from stems and twig cankers, for example, *Botryosphaeria dothidea*, *Macrovalsaria megalospora* (Mont.) Sivan., *Nattrassia mangiferae* (Mont.) Sivan., *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *Lasiodiplodia theobromae* (Old et al. 2000; Nghia 2010, 2015).

2.4.3 Vascular wilts and cankers

The most important group of vascular wilt fungi of plantation tree species, termed 'ophiostomatoid fungi' on account of their morphological similarity, are represented by genera in the Ascomycota families Ceratocystidaceae and Ophiostomataceae (Wingfield et al. 2017). These fungi are commonly associated with tree-colonizing insects. Dutch elm disease (DED), caused by the fungi *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*, is considered one of the most devastating tree vascular wilt diseases in the world (Pecori et al. 2017). *Ceratocystis* is a genus of fungi that contains virulent pathogens of a wide range of plants (Table 2-2) and have been reported to cause disease on various types of *Acacia* species (Table 2-3).

Table 2-2: *Ceratocystis* spp. reported to cause disease in trees worldwide. Note that different authors have differing views on the correct name for the pathogen infecting mangoes and Acacias.

Host	Pathogen	Country	Reference
Dalbergia sissoo	*C. manginecans*	Pakistan	(Al Adawi et al. 2013b)
Eucalyptus spp.	*Ceratocystis fimbriata*	Brazil, Africa, Congo, Uganda	(Zauza et al. 2004; Harrington et al. 2015)
E. obliqua	*C. moniliformopsis*	Australia	(Yuan & Mohammed 2002)
E. obliqua	*C. eucalypti*	Australia	(Kile et al. 1996)
Eucalyptus spp.	*C. neglecta*	Colombia	(Rodas et al. 2008)
Eucalyptus spp.	*Ceratocystis* spp.	Ecuador	(van Wyk et al. 2011)
Mangifera indica	*C. manginecans*	Oman and Pakistan	(Wyk et al. 2007; Al Adawi et al. 2013a; Oliveira et al. 2015)
M. indica	*C. fimbriata*	Brazil	(Oliveira et al. 2015; Araujo et al. 2016)
Metrosideros polymorpha	*C. fimbriata*	Hawaii	(Keith et al. 2015)

Host	Pathogen	Country	Reference
Picea engelmannii *P. glauca*	*C. coerulescens*	North America	(Wingfield et al. 1997)
P. abies	*C. polonica*	Norway	(Zhao et al. 2010)
Prosopis cineraria	*C. manginecans*	Oman	(Al Adawi et al. 2013b)
Platanus	*C. platani*	Italy	(Panconesi 1981)
Quercus spp.	*C. fagacearum*	USA	(Haight et al. 2011; Harrington 2013)

Table 2-3: *Ceratocystis* spp. isolated from *Acacia* plantations worldwide.

Host	Pathogen	Country	Reference
Acacia mangium	*Ceratocystis* spp.	Vietnam	(Thu et al. 2012)
A. auriculiformis, *Acacia* hybrid	*C. manginecans*		(Thu 2016b)
A. mangium	*C. inquinans*	Indonesia	(Tarigan et al. 2010)
	C. mikrobasis		
	C. sumatrana		
A. mangium, *A. crassicarpa*	*C. manginecans* (syn. *C. acaciivora)*	Indonesia	(Tarigan et al. 2011b)

Host	Pathogen	Country	Reference
A. mearnsii, *A. decurrens*	*C. albifundus*	Uganda, South Africa, Kenya, Tanzania	(Morris et al. 1993; Roux & Wingfield 1997; Heath et al. 2010) (Wingfield et al. 1996; Roux et al. 1999)
A. decurrens	*C. fimbriata*	Brazil	(Ribeiro et al. 1988)

The taxonomy of *Ceratocystis* is complex and the genus incudes several species complexes (Harrington et al. 2014). A recent taxonomic revision has elevated some of these species complexes to the level of genus; e.g. the *C. coerulescens* (Münch) B.K. Bakshi complex was transferred to *Endoconidiophora*, *Thielaviopsis* was emended to include the *C. paradoxa* (Dade) complex and the new genus *Huntiella* erected to accommodate the *C. moniliformis* (Hedgc.) complex, (de Beer et al. 2014). The large *C. fimbriata* Ellis & Halst. complex remains in *Ceratocystis* as it is the type species for this genus.

Ceratocystis manginecans (*Acacia* canker and wilt) is in the *C. fimbriata sensu lato* species complex, which is an assemblage of morphologically similar and phylogenetically closely related species. *Ceratocystis manginecans* used to be considered as a closely related species to *C. acaciivora*, which causes canker and wilt disease of *A. mangium* in Indonesia (Tarigan et al. 2011a). However, *C. manginecans*

and *C. acaciivora* were reduced to synonymy based on DNA analysis with the name *C. manginecans* taking precedence (Fourie et al. 2015).

The disease symptoms caused by *Ceratocystis* spp. vary depending on the type of hosts (Barnes 2002) and include die-back, gummosis, wilting (Wingfield et al. 1996; Roux et al. 1999), staining of the vascular tissue and cankers (Wyk et al. 2007). The external symptoms of *Ceratocystis* canker and wilt disease in *A. mangium* typically include cracked or sunken bark above cankers, blackened streaks (ray like stain) within the vascular tissue, gummosis or exudation and fungal fruiting bodies (Tarigan et al. 2011a; Brawner et al. 2015).

All *Ceratocystis* spp. on woody hosts infect through wounds and move through the sapwood tissue, mostly in ray parenchyma (hence the ray like stain pattern) and some also cause cankers (Harrington 2009). Therefore, for those pathogen species to invade living trees the presence of wounds is a prerequisite. Wounds created by pruning and thinning activities (Tarigan et al. 2011b) as well as damage by animals including elephants (Kamgan et al. 2008), monkeys (Tjahyono pers. comm.) or insects (Wingfield 2017) are potential infection courts for *Ceratocystis*. *Ceratocystis* can also be disseminated by root grafting (Bruhn et al. 1991; Wilson & Lester 2002; Juzwik et al. 2010). Inoculum can also survive in soil and infect through the roots (Ferreira et al. 2013).

Ceratocystis species produce a range of different spore types and each is associated with various dissemination methods. The ascospores are produced in a sticky mass

that is ideal for adhering to insects (Dowding 1984; Tarigan et al. 2012). Asexual conidia are also commonly produced and in some species, the darkly pigmented, thick-walled aleurioconidia or chlamydospores (de Beer et al. 2014). The aleurioconidia are produced in woody substrates (Ferreira et al. 2013) and may be important for survival in soil and water (Grosclaude et al. 1991).

Ceratocystis species may be vectored by wood-and bark-associated insects, typically those that visit fresh wounds acting as infection courts (Heath et al. 2009; Al Adawi et al. 2013b). The insects inadvertently pick up *Ceratocystis* spores from infected trees, subsequently visiting trees with fresh wounds and depositing spores as they feed on the sap (Moller & Devay 1968). Lin & Phelan (1992) showed that *C. fagacearum* (Bretz) J. Hunt can produce fruity odours including 16 components: one aldehyde, one ketone, five alcohols and nine esters, many of which were previously shown to be attractive to nitidulid beetles. Thus, by mimicking food odours, *C. fagacearum* has adapted to attract nitidulid and possibly other insect vectors.

Wood-boring beetles such as *Hypocryphalus mangiferae* (Al Adawi et al. 2013b) and *Euwallacea interjectus* (Kajii et al. 2013) can also carry spores of *Ceratocystis* species. In addition to carrying spores on their bodies, they contribute to wind-dispersed inoculum in the form of frass (Jeger et al. 2016). As the beetles tunnel through infected wood, a tube of frass containing conidia and/or chlamydospores is ejected from the tunnel, facilitating dispersal by wind (Lin & Phelan 1992; Jeger et al. 2016).

Since the appearance of *Ceratocystis* in SE Asian *Acacia* plantations, pathogenicity tests have been conducted on a number of *Acacia* species (Tarigan et al. 2011a). An artificial inoculation trial in Vietnam tested 26 *C. manginecans* isolates on 8-month old potted *A. mangium* seedlings (Thu et al. 2012). Based on lesion development and tree death, 13 isolates were highly pathogenic, causing plant death, two isolates were not pathogenic and the other 11 isolates produced symptoms ranging from low to moderate severity.

Fusarium euwallaceae S. Freeman, Z. Mendel, T. Aoki & O'Donnell is another fungal species that causes vascular wilt in a broad range of tree species (Freeman et al. 2013). Vectored by the beetle *Euwallacea fornicatus*, it has become an invasive pest in Israel and California, infecting over 20 tree species but particularly devastating to avocado orchards (Freeman et al. 2013). Both beetle and fungus are thought to represent species complexes which originate from SE Asia (O'Donnell et al. 2015; O'Donnell et al. 2016) though their native host and geographical ranges are poorly known. *Fusarium euwallaceae* has been isolated from *Acacia* plantations in Vietnam, with up to 50 % mortality attributed to *F. euwallaceae* in some locations (Ha 2015).

2.4.4 Identification of fungi associated with heart rot, cankers and wilts

2.4.4.1 Identification by morphological techniques

Accurate and robust detection and identification of fungal pathogens are essential for all aspects of plant pathology including the biology of pathogens, diagnosis, modelling, surveillance (Tsui et al. 2011) to the control of diseases (McCartney et al. 2003). Morphological identification is based on a comparison of morphological characteristics with herbarium specimens and published descriptions (Wingfield et al. 1996). However, this method requires highly specialised and experienced people and even so, it may be very difficult to distinguish between and/or among some species. As an example, some species in the Ceratocystidaceae are very similar to each other; e.g. *Huntiella chinaeucensis* (S.F. Chen bis, Jol. Roux, M.J. Wingf. & X.D. Zhou) Z.W. de Beer, T.A. Duong & M.J. Wingf.) is morphologically similar to *H. inquinans* (Tarigan, M. van Wyk & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *H. microbasis* (Tarigan, M. van Wyk & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *H. omanensis* (Al-Subhi, M.J. Wingf., M. van Wyk & Deadman) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *H. sumatrana* (Tarigan, M. van Wyk & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Chen et al. 2013).Wood decay fungi including heart rot fungi can sometimes be identified from the morphological and physiological characteristics of fruiting bodies (Lee & Noraini Sikin 1999). Based on the fruiting bodies produced, four wood decay fungi (*Rigidoporus hypobrunneus* (Fetch) Corner, *Phellinus noxius* (Corner) Cunn., *Tinctoporellus epimiltinus* (Berk. & Br.) Ryv. and *Oxyporus* cf. *latemarginatus* (Dur.

& Mont. *ex* Mont.) Donk) isolated from *A. mangium* in Malaysia have been identified (Lee & Noraini Sikin 1999). In addition to being very time-consuming, this method of fungal identification has been hampered by the absence of fruiting bodies or sporocarps on affected trees and the failure of sporocarp production *in vitro* (Lee & Noraini Sikin 1999).

Wood decay fungi can also be identified from the morphological and physiological characteristics of the mycelia in culture by comparison with the species codes developed by Nobles (1948) and Stalpers (1978). By using this technique, Lee & Maziah (1993) identified *Phellinus noxius* associated with heart rot in seven-and eight-year-old *A. mangium* trees in Peninsular, Malaysia. Mehrotra et al. (1996) identified *Phellinus pachyphloeus* (Pat.) Pat. and *Trametes palustris* (Berk. & M.A. Curtis) Ryvarden (syn. *Fomitopsis palustris* (Berk. & M.A. Curtis) Gilb. & Ryvarden) from decayed wood of seven- to ten-year-old *A. mangium* trees in West Bengal, India. However, due to the limitation of species codes and the limited range of characterised fungi, mainly of temperate species in Nobles (1948) and Stalpers (1978), many isolates associated with *A. mangium* decayed wood remained unidentified (Lee & Maziah 1993).

2.4.4.2 Fungal identification by molecular techniques

Although prevalent traditional techniques relying upon culture-based morphological approaches are useful to identify plant pathogens in many situations, there are many fungi which are difficult or impossible to cultivate (Tsui et al. 2011), lack

morphological characteristics or distinguishing microscopic features in culture or fail to produce sporocarps (Nobles 1948). Additional problems include the lack of keys and accessible type specimens, misapplication of European names for tropical and subtropical taxa (Cao et al. 2012) that make identification of plant pathogens and other fungi impossible using traditional techniques. In addition, these techniques are often time-consuming, laborious and require extensive knowledge of classical taxonomy (Capote et al. 2012). These limitations have led to the development of molecular approaches with improved accuracy and reliability (Capote et al. 2012).

Molecular methods for the identification of plant pathogens have been developed and applied since the 1980's when species-specific DNA probes were applied to Southern blots (Henson 1989). The development of the polymerase chain reaction enabled species identification by species-specific PCR and this continues to be widely applied, such as for the detection and identification of *Mycosphaerella* spp., common causal agents of leaf spot diseases on *E. globulus* and *E. nitens* in Australia (Glen et al. 2007), *Ganoderma* species associated with root disease in *A. mangium* and *E. pellita* (Yuskianti et al., 2014) and *C. fagacearum*, causal agent of oak wilt (Wu et al. 2011). Quantitative or real-time PCR was a subsequent refinement. Wu et al. (2011) developed a nested, real-time PCR to detect *C. fagacearum* in stained wood and soil by analysing ITS sequences of *Ceratocystis* spp.; *Ceratocystis platani*, the causal agent of canker stain of *Platanus* spp. was detected by real-time PCR techniques based on the use of an intercalating dye, EvaGreen, and a Taq-man probe (Pilotti et al. 2012; Luchi et al. 2013). The major disadvantage of species-specific PCR is that it targets a single fungal species, or a small group of related species,

whereas heart-rot often involves a suite of causal organisms and requires comprehensive testing of species-specificity (Lee et al. 1988).

Current techniques for molecular identification of fungi are mainly based on the comparison of DNA sequence data. This approach, termed DNA barcoding, has been applied to many organisms (Hebert et al. 2016; Hollingsworth et al. 2016; Miller et al. 2016). The ITS region has been accepted as the primary DNA barcode marker for fungi (Schoch et al. 2012), though this region is recognised as not ideal for several reasons (Kiss 2012). Supplementary barcodes are used for many classes of organism (Schoch et al. 2012; Hollingsworth et al. 2016). Supplementary barcodes that have proven useful in discriminating fungal species include β-tubulin and the transcription elongation factor 1-α gene regions (van Wyk et al. 2009; Oliveira et al. 2015). In addition, this method of identification can be based on other gene regions such as the 28S nuclear ribosomal large subunit rRNA (LSU) or the 18S nuclear ribosomal small subunit rRNA gene (SSU) sequence (Borman et al. 2008; Schoch et al. 2012), where a comprehensive database of sequences can be generated from robustly identified specimens. The advantage of using molecular data is that it provides a greater number of heritable characters that allow for convenient information sharing between laboratories (Cai et al. 2011).

Fungal identification is inextricably linked to taxonomy, which has undergone a major revision as natural lineages are revealed by DNA-based phylogenetic analysis (Voigt & Kirk 2011). Initially relying to a great extent on ribosomal DNA sequence data, fungal systematists now recognise the need for multigene phylogenetic analysis

to avoid creating a gene phylogeny rather than a species phylogeny (Taylor et al. 2000; Cai et al. 2011).

Protein-coding genes are widely used in mycology for phylogenetic analyses or species identification (Schoch et al. 2012). Different genes such as translation elongation factor 1-α have been applied to the identification and discrimination of *Fusarium* species (O'Donnell et al. 2010) and β-tubulin for *Penicillium* (Frisvad & Samson 2004) and several *Ceratocystis* species (van Wyk et al. 2009; Oliveira et al. 2015). However, primers for these regions may amplify DNA from a narrow taxonomic range (Schoch et al. 2012).

Phylogenetic approaches based on variable characters, usually DNA sequences of selected genes or genomes (Cai et al. 2011) are also being used to identify taxonomic units of fungi such as in the genera *Agaricus* (Geml et al. 2008), *Ganoderma*, *Cerrena*, *Tinctoporellus* (Glen et al. 2014). Although phylogenetic approaches have some weaknesses (Aguileta et al. 2008), those techniques have become a routine procedure in identifying fungal species (Zhou et al. 2016; Raja et al. 2017).

Although the identification of fungal pathogens based on molecular methods has many advantages, it still poses some problems. For example, a lack of confidence in accurate identification of specimens from which sequences in the INSDs (International Nucleotide Sequence Databases) were derived was a major obstacle to confidence in identification. Confirmation of a potential barcode gap for other species was made more difficult by the occurrence of probably misidentified

accessions in the INSDs (Glen et al. 2014), and only a very small number of species have been deposited in public culture collections and only a fraction of these have had some DNA fragment sequenced (Cai et al. 2011). While some curated fungal databases are available, these are, as yet, insufficiently populated to allow identification of all fungi so use of the INSDs can be helpful as long as care is taken to avoid the many potential pitfalls.

DNA bar-coding approaches to fungal identification have paved the way for cultivation-free methods of assessing ecological roles of fungi. This is termed environmental sequencing or metagenomics (Hibbett et al. 2016) and this has been given a significant boost by the availability of next generation sequencing technologies (Metzker 2010; Buermans & den Dunnen 2014). The combination of high throughput sequencing and bioinformatics allows rapid and robust characterisation of fungal species in complex communities such as rotting wood (Studholme et al. 2011; Dannemiller et al. 2014). Consideration is even being given to the delineation of new fungal species based entirely on DNA sequences in the absence of specimens or isolates (de Beer et al. 2016).

2.5 Screening for resistance

Plants respond to pathogen attacks by erecting a highly coordinated series of molecular, cellular and tissue-based defence barriers (Guest & Brown 1997). In plants, passive or constitutive defence mechanisms are present before contact with the pathogen, whereas active or induced defence mechanisms occur only after

pathogen recognition. To gain access to the nutrients in plants, pathogens must breach the natural barriers (physical or chemical) presented in healthy plants. Many plant secondary metabolites present effective chemical barriers due to their antifungal activity (Osbourn 1996). Some of these compounds are constitutive, presenting in tissue before colonization by pathogens (Bonello et al. 2006), while others occur as inactive precursors and are activated in response to pathogen attack (Osbourn 1996).

Planting of resistant genotypes is the most suitable control strategy for forest plantation diseases given the extensive planting of monocultures and the relatively low value of each economic unit (tree) (Guimaraes et al. 2010b; Alfenas et al. 2016). Tree improvement programs focus on improving growth rate, stem form, wood properties (Cameron et al. 2012; Harwood et al. 2015; Missanjo & Matsumura 2016) and may also consider disease resistance at their inception (Dudley et al. 2015; Harwood et al. 2015; Liu et al. 2017). Screening for and successful deployment of disease-resistant genotypes has been applied in breeding programs for forest trees such as *E. grandis* (Junghans et al. 2003), *E. pellita* (Guimaraes et al. 2010b), *E. brassiana*, *E. saligna*, *E. scias* and *E. agglomerata* (Alfenas et al. 2016), *Acacia koa* (Dudley et al. 2015), *Picea abies* (Mottet et al. 2015), *Acacia* hybrid and *Eucalyptus* spp. (Nghia 2006, 2010).

2.5.1 Molecular tools for resistance screening

Molecular techniques have provided tools to characterise all stages of the interaction between plants and potential pathogens for over two decades (Capote et al. 2012; Raja et al. 2017). These techniques have been applied to identification of disease resistant genotypes of forest trees, e.g. using RAPD (random amplified polymorphic DNA) markers to screen for *Eucalyptus grandis* resistant to *Austropuccinia psidii* (Junghans et al. 2003), mapping of quantitative trait loci (QTLs) for resistance to *Ceratocystis* (Rosado et al. 2016) and *Austropuccinia psidii* (Butler et al. 2016), the identification of pathogenesis-related proteins in *E. grandis* such as PR-1, PR-2, PR-3 (Naidoo et al. 2014) or using identical-by-descent based methodologies to screen for *E. grandis* x *E. urophylla* populations resistant to *P. psidii* (Rosado et al. 2010).

Such methods are dependent upon knowledge of the host tree genetics and characterisation of loci influencing disease susceptibility. The availability of a sequenced genome, as is the case for *E. camaldulensis* and *E. grandis*, but not yet for *Acacia mangium* and its hybrids (Hirakawa et al. 2011; Myburg et al. 2014), provides resources to extend our understanding of tree defences (Naidoo et al. 2014).

2.5.2 Biochemical tools and resistance screening

Constitutive and induced antimicrobial compounds have been investigated in plantation trees, both gymnosperms and angiosperms (Schmidt et al. 2005; Eyles et al. 2010). Terpenoids and phenolics are present in almost all plants, whereas

alkaloids and other nitrogen-containing secondary metabolites are more common in angiosperms (Wink 2016). The low-molecular-weight (LMW) compounds involved in defence are classified according to their biosynthetic pathways, including terpenoids, phenolic compounds [>8,000 known structures from the phenylpropanoid pathway (Keeling & Bohlmann 2006)] and alkaloids [>12,000 known structures from the alkaloid pathway (Facchini 2001)].

Phenolic chemistry has been much studied in woody plants for determining markers of resistance (Witzell & Martín 2008) or susceptibility to pathogens (Woodward et al. 2007). Such studies have been undertaken in pines (Blodgett et al. 2007; Sherwood & Bonello 2013), eucalypts (Eyles et al. 2003a), Sitka spruce (*Picea sitchensis*), oaks (McPherson et al. 2014), *Acacia confusa* (Chen et al. 2014) and also in fruit trees such as mango (Araujo et al. 2014; Araujo et al. 2016).

Constitutive phenolic compounds or pre-existing low-molecular-weight (LMW) antimicrobial compounds are named phytoanticipins (van Etten et al. 1994) while induced phenolic compounds or LMW antimicrobial compounds that synthesised *de novo* upon infection are described as phytoalexins (Hammerschmidt 1999; Bonello et al. 2006). Phytoalexins consist of an extremely diverse group of secondary metabolic compounds including flavonoids, pterocarpans, stilbenes, lignans, tannins and saponins (Hammerschmidt & Nicholson 1999; Witzell & Martín 2008). The distinction between constitutive antimicrobial compounds and phytoalexins is not always clear (Huang 2001). It is, however, important to know that the distinction between a phytoalexin and a phytoanticipin is not based on its chemical structure but

rather on how it is produced, therefore the same chemical may serve as both a phytoalexin and a phytoanticipin, even in the same plant (VanEtten et al. 1994).

Tannins and flavonoids appear to be major classes of inducible phenolics in many hardwood woody species (Witzell & Martín 2008) and they have been demonstrated as inhibitory to pathogenic fungi in woody plants (Pearce 1996; Zeneli et al. 2006). Pre-existing phenolic compounds such as flavan-3-ols and proanthocyanidins were induced in the hybrid plane (*Platanus* x *acerifolia*) inoculated with *Ceratocystis fimbriata* f. sp. *platani* (El Modafar et al. 1996). The rapid accumulation of these compounds near the inoculation site, especially in the medulla, vascular tissues, tyloses, and gels, indicated that these chemical responses were of a defensive nature (El Modafar et al. 1996). Hydrolysable tannins, polymeric proanthocyanidins, flavonoid glycosides, formlyated phloroglucinol compounds and volatile terpenes have been detected in the margins of lesions inoculated with the canker fungus *Cytonaema* sp. (Eyles et al. 2003b). These chemicals may also be induced by wounding in *E. globulus* and *E. nitens* (Eyles et al. 2003a).

Flavonoids and condensed tannins have been much studied in the heartwood of *Acacia* species, driven by the need to understand its pulping properties and also by the pharmaceutical and food industries in search of new bioactive molecules. Flavonoids are low molecular weight compounds and comprise a large group of polyphenolic compounds having a benzo-γ-pyrone structure (Figure 2-1) that are ubiquitously present in plants (Kumar & Pandey 2013). Flavonoids are known to be synthesised by plants in response to microbial infection (Dixon et al. 1983). In

A. mangium healthy heartwood, two major flavonoids have been reported; these are 2,3 *trans*-3,4',7,8-tetrahydroxyflavanone and 4,7,8-trihydroxyflavanone (Pietarinen et al. 2004). In *A. auriculiformis* heartwood, in addition to the two major flavonoids reported in *A. mangium*, eight flavonoids have been identified, all having the 4',7,8-hydroxylation pattern, and include isoteracacidin (the 2,3-cis-3,4-trans isomer) (Drewes & Roux 1966).

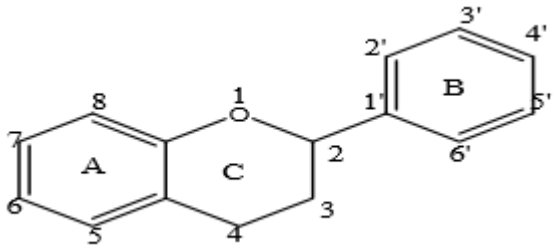
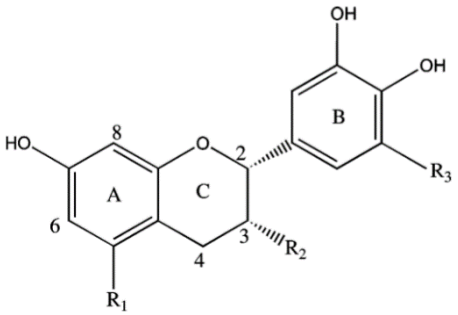


Figure 2-1: Basic structure of flavonoids (Sandhar et al. 2011)

Condensed tannins have been found in *A. mangium* and *A. auriculiformis* heartwood with reference to studies of heart rot susceptibility (Barry et al. 2005b; Mihara et al. 2005). Condensed tannins which consist of a group of polyhydroxyflavan-3-ol oligomers and polymers linked by carbon-carbon bonds between flavanol subunits (Schofield et al. 2001) (Figure 2-2 and Figure 2-3) are the most conspicuous secondary compounds in most species of *Acacia* i.e. procyanidins, profisetinidins, prorobinetinidins, prodelphinidins, and roteracacinidins (Foo 1984; Seigler 2003). *Acacia* heartwoods are dominated by condensed tannins and flavonoids with similar hydroxylation patterns (Foo 1984).



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 2-2: The basic repeating unit in condensed tannins. If R1 =R2 = OH, R3 = H, then the structure is that for (-)-epicatechin. The groups at R1 and R3 for other compounds are indicated below the structure. R2 = O-galloyl in the catechin gallates (Schofield et al. 2001).

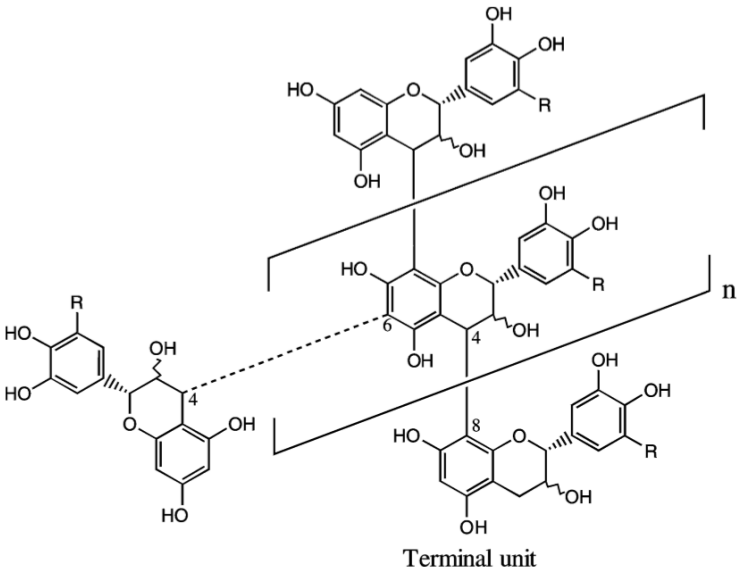


Figure 2-3: Model structure for a condensed tannin. If R = H or OH then the structure represents a procyanidin or prodelphinidin. The 4 – 6 linkage (dotted line)

is an alternative interflavan bond. The terminal unit is at the bottom of such a multi-unit structure (Schofield et al. 2001).

A higher extractive content, especially of the condensed tannin, teracacidin, was found in the heartwood of *A. auriculiformis* compared with *A. mangium* (Barry et al. 2005b; Mihara et al. 2005). As *A. auriculiformis* is less susceptible than *A. mangium* to *Ceratocystis* (Tarigan et al. 2016) as well as heart-rot (Barry et al. 2005b), it would be of interest to further investigate the antifungal chemical responses of hybrids of *A. mangium* and *A. auriculiformis* (Mihara et al. 2005) with a focus on flavonoids and condensed tannins.

2.6 Managing stem defect for solid wood production in *Acacia* – the literature to recommendations

This review has shown that silvicultural techniques such as pruning and thinning are essential in managing forest plantations grown for solid wood, especially for *Acacia* and *Eucalyptus* plantations. Thinning can increase the volume of a stand, while pruning can improve the quality of solid logs. Despite the advantages of pruning and thinning, these techniques may also have negative impacts on plantations as they are a pathway for heart-rot fungi to infect the trees via wounds and subsequently cause heart rot and stem defects.

Control of branch size is of vital consideration in the silviculture of plantations managed for solid wood due to the association between pruning larger diameter

branches and fungal infection (Mohammed et al. 2000; Beadle et al. 2008). There are two common management options, stocking rate and form pruning (Beadle et al. 2007; Sein & Mitlohner 2011). Higher initial stocking densities will reduce the incidence of large branches (Neilsen & Gerrand 1999).

Pruning should be done with great care in order to avoid damage to the branch collar, the branch bark (Sein & Mitlohner 2011) and also the stem bark, as such damage can lead to disease. Pruning tools should always be cleaned and sharpened to ensure a clean, smooth cut (Sein & Mitlohner 2011). Tarigan et al. (2012) showed that careful pruning can reduce the incidence of stem disease caused by *Ceratocystis mangenicans* in *Acacia* plantations by 50%. Excessive pruning and rough pruning practices should be avoided. The application of systemic pesticide leads to lower disease incidence compared to controls, under both careful and rough singling practices (Tarigan et al. 2012). However rapid growth of the trees in sub-tropical and tropical *Acacia* which would very soon cause cracks to appear in the wound treatment barrier thereby rendering it ineffective (Lee & Maziah 1993). More importantly the treatment of pruning wounds with a fungicide or sealant is cost prohibitive given the potential number of trees and wounds requiring treatment and could only be considered for high value trees such as those in seed orchards.

As described in section 1.3.2 Beadle et al. (2013b) have developed pruning prescriptions for lift pruning and tip pruning for *Acacia* hybrids in Vietnam which also target the reduction of fungal infections. Lift pruning is to be undertaken only in the dry season to minimise the risk of disease entry through pruning wounds adjacent

to the stem. Tip pruning removes the dominance of competing stems but growth potential is retained and excision of unhardened stems/branches at the point where they join the retained stem is avoided, theoretically reducing the potential for disease entry. Unfortunately there has been little research into the management of thinning to reduce potential stem defects although this thesis shows that thinning can influence the incidence of stem decay (Trang et al. 2017).

Selecting and planting trees resistant and tolerant to diseases is a widely used management strategy, especially in forestry. Experience in rubber plantations has shown that planting of disease-resistant varieties would be the best strategy to prevent the occurrence of pink disease (Old et al. 2000). Guimaraes et al. (2010b) stated that "planting of resistant genotypes is the most suitable control strategy of forest diseases under field condition" and for vascular wilt diseases, the most effective control strategy is the use of genetic control (Yadeta & Thomma 2013).

Nguyen (1999) showed that the most significant diseases on eucalypts in Vietnam are *Cryptosporiopsis eucalypti* Sankaran & B. Sutton, *Cylindrocladium quinqueseptatum* (syn. *Calonectria quinquesepta* Figueiredo &Namek.) and identified some best performing eucalypt provenances, in term of growth and disease resistance e.g. clones EF24, EF39 and EF55. Other research in Vietnam (Nghia 2006, 2010) showed that there are some *Acacia* clones that have greater disease resistance to pink disease i.e. clones of *A. mangium* (e.g. clones AM2, AM3), *A. auriculiformis* (e.g. clones AA1 and AA9) and hybrids (e.g. clones AH1 and AH7) of the two species.

Different provenances appear differentially impacted by heart-rot (Ito & Nanis 1994). Provenance differences in branching may have an influence on heart-rot incidence (Barry et al. 2004). There is potential for heart-rot incidence to be reduced by utilising *A. mangium* trees which have been bred for single stems, small branches and early branch shedding (Arnold & Cuevas 2003).

Since the discovery of *Ceratocystis manginecans*, there has been a series of resistance screening trials with *Acacia* in Indonesia, Malaysia and Vietnam (Tarigan et al. 2011a; Thu et al. 2012; Chen et al. 2013; Brawner et al. 2015; Tarigan et al. 2016). While levels of tolerance to *C. manginecans* in *A. mangium* are low and resistance is rarely observed, other species such as *A. auriculiformis* show greater tolerance (Trang et al. 2018). The research in this thesis confirmed that a clear gradient of tolerance to *C. manginecans*, as indicated by lesion lengths, exists in *Acacia* species. This variation must be fully exploited, especially the transference of tolerance from *A. auriculiformis* to *A. mangium* through hybridisation.

The literature review indicates that the successful management of heart rot and *Ceratocystis* in plantations for solid wood production will require a transdisciplinary and team approach. Expertise in practical silvicultural methods, tree physiology, tree genetics, host defence and the interactions of all these parameters with fungal pathogen biology and the expression of disease will be required.

Chapter 3

Quantifying stem discoloration and decay following pruning and thinning an *Acacia* hybrid plantation

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Summary

Acacia hybrid (*Acacia mangium* × *A. auriculiformis*) is widely planted in Vietnam and part of the estate is managed for solid timber products. This requires pruning and thinning, practices that through mechanical wounding can facilitate the entry of fungal organisms, leading to stem defects. The extent to which this happens in *Acacia* hybrid has not been previously studied in Vietnam. A destructive survey was conducted in a 3-year-old *Acacia* hybrid plantation at Nghia Trung in Binh Phuoc province, 18 months after the imposition of pruning and thinning treatments. Pruned trees had a higher incidence and severity of discoloration and decay in the stem than unpruned trees; thinning increased the incidence of stem decay and the severity of discoloration but not the severity of decay. An interaction between pruning and thinning did not influence the incidence of stem decay but did increase the severity of discoloration in thinning treatments. Across treatments, levels of discoloration and decay were <30% and <5%, respectively. Modification of current pruning practices may be necessary to ensure that decay levels are kept within acceptable limits at an anticipated harvest age of 7-8 years.

3.1 Introduction

The heartwood in *Acacia mangium* Willd. is reported highly susceptible to fungal decay (Mahmud et al. 1993; Barry et al. 2002b; Bougher & Tommerup 2002), whereas that of *Acacia auriculiformis* A.Cunn. ex Benth. is considered resistant (Ito 2002). Surveys conducted in Malaysia showed that the natural hybrid between these two species was susceptible to heartwood rotting fungi, but less so than *A. mangium* (Lee 2004). A more recent study confirmed that the heartwood of *Acacia* hybrid may have some resistance to fungal decay (Rokeya et al. 2010). By 2011, approximately 230,000 ha of the *Acacia* hybrid had been planted in Vietnam and this area accounted for 30% of the country's total plantation estate of tropical acacias (Griffin et al. 2011). While *Acacia* hybrid is harvested mainly for export woodchips, a significant proportion is now being managed for solid-wood production, mainly for furniture (Kha et al. 2012). If this is to be successful any discoloration and decay in the stem will need to be minimised.

Plantation management for solid-wood production includes practices such as pruning which leads to the production of knot-free wood (Barry et al. 2005a) and logs of sufficient quality for sawing (Gerrand et al. 1997). Thinning enables selection of trees with the best form (Beadle et al. 2007; Beadle et al. 2013a), and increases the growth rates of individual trees (Wood et al. 2009; Forrester et al. 2010), saw-log volume (Gerrand et al. 1997; Wardlaw 2003; Medhurst et al. 2011) and saw-log value (Beadle et al. 2013a). However, both pruning and thinning can increase the risk of stem defects (Han & Kellogg 2000; Barry 2002) and create

infection courts for fungi (Medhurst et al. 2002; Pinkard et al. 2004). For example, in *Eucalyptus nitens* (Deane and Maiden) Maiden, 13.5-16.2% of pruned living branches were associated with columns of discoloration and decay spreading from the pruned stub into the stem (Wardlaw & Neilsen 1999). Hunt & Krueger (1962) found that 61 and 23% of the wounds caused by thinning plantations of *Tsuga heterophylla* (Raf.) Sarg. and *Pseudotsuga menziesii* (Mirb.) Franco, respectively, were associated with decay; in 19-20-year-old thinned stands of *Eucalyptus regnans* F.Muell., 8.7-15.7% of wounds were associated with discoloration and decay (White & Kile 1991). Injuries caused by branch removal in plantations after wind events, self-pruning, and also injury-related diseases such as stem cankers can also lead to decay inside living trees (White & Kile 1991; Pinkard et al. 2004). Decay fungi cause typical white-rot symptoms leading to changes in the colour, texture and quality of wood in plantation tree species (Lee et al. 1988; Mahmud et al. 1993; Pearce 1996). These changes are used to assess the levels of incidence and severity of heart rot on harvested log-ends in the field (Pyle & Brown 1998; Mohammed et al. 2006; Grove et al. 2011).

Heart rot is fungal decay in the central stem or heart wood, though some fungi also cause sapwood rot. When functional sapwood is killed by a decay fungus this is called a canker rot but the same fungus may simultaneously cause heart and sapwood rot (Vasaitis 2013). There are four different concepts of infection and colonisation of a tree by fungi (Manion 1990): i) the ‘Haddow-Etheridge’ concept, which describes latent establishment (Haddow 1938; Etheridge & Craig 1976); ii) the ‘Hartig’ concept of fungi that infect a tree via a wound that exposes heartwood

(Hartig 1874); iii) the 'Shigo' concept of fungal succession after entry via sapwood wounds (Shigo 1966); and iv) the 'Boddy-Rayner' concept explaining the importance of moisture content in wood decay patterns (Boddy & Rayner 1983) and later, the role of endophytic fungi as potential sources of decay (Boddy & Rayner 1983). Vasaitis (2013) categorises the fungi that infect living trees as: i) true heart-rot fungi that gain entry via natural infection courts; ii) true heart-rot fungi that infect via natural wounds such as fire scars or broken branches; and iii) wound heart-rot fungi that enter through dead sapwood and comply to the Shigo concept. In this study, decay is defined as the decomposition of heartwood inside a living tree. Fungi could have gained entry by all three of the above (Vasaitis 2013) but for the trees that were pruned and thinned in this study, the predominant type of fungi responsible for decay is assumed to be "wound heart rot". Discoloration is defined as "an early stage of decay where decomposition remains absent" (Mahmud et al. 1993).

To date, there have been very few studies that have examined the effect of silvicultural treatment on stem discoloration and decay for tropical plantation species. In this paper, we report the results from a study undertaken in a young, intensively managed, *Acacia* hybrid plantation at Nghia Trung in Binh Phuoc province, southern Vietnam, which was being managed for solid-wood production. Live branches on trees were either pruned to 1.5 m or left unpruned. The following hypotheses were tested: below 1.5 m tree height (i) pruned trees have a higher incidence and severity of discoloration and decay than unpruned trees, (ii) trees in the thinned treatment have a higher incidence and severity of discoloration and

decay than in the unthinned treatment, (iii) a thinning and pruning interaction increases the incidence and severity of discoloration and decay; and (iv) above 1.5 m tree height, pruned trees have a higher severity of discoloration and decay than unpruned trees. In analysing the results, it was important to clearly differentiate between discoloration and decay as each has different implications for the potential end-use of the wood.

3.2 Materials and methods

3.2.1 Site and experimental design

The *Acacia* hybrid plantation at Nghia Trung was established in late July 2009 at 1111 stems ha^{-1} (3 m x 3 m). The site had been previously planted with a pulpwood plantation species, *Aquilar crassna* Pierre in 2005; however most of these had died and been replaced by volunteer *Acacia mangium* wildings. The *Acacia* hybrid planting stock consisted of a randomised mixture of four clones, TB01, TB06, TB11 and TB12 hybrids which had been developed from open-pollinated *A. mangium* female parents with the male parent being *A. auriculiformis*. The ramets were planted into 40 wide × 40 long × 40 cm deep planting pits. Phosphorus fertiliser in the form of superphosphate equivalent to 50 kg P ha^{-1} had been placed at the bottom of the pit just prior to planting. Round-up at 4 L ha^{-1} (0.48 g L^{-1} and 1.92 kg ha^{-1} glyphosate isopropylamine salt) was applied with a shrouded sprayer on four occasions, once before planting, and at ages 2.5, 9 and 16 months, the last one month before the application of fertiliser at thinning at age 17 months. To maximise the development of trees with straight stems, the trees were “singled” at

age four months by removing any stems competing with the selected leader, and form pruned at ages 12 and 16 months by removing 50% of the length of branches of diameter >3 cm. This type of form pruning is important for *Acacia* species (Medhurst et al. 2006); *Acacia* hybrid exhibits only moderate apical dominance.

There was a total of three randomised complete blocks, with 6 plots each, hence a total of 18 plots. Each block comprised the following: two thinning treatments, an unthinned control and thinned to 600 stems ha^{-1}; three fertiliser treatments at thinning, no fertiliser, 50 kg P ha^{-1} and 50 kg P ha^{-1} + basal fertiliser, giving a total of 6 treatment combinations. The basal fertiliser contained salts of all other macronutrients (K, Mg) except N and Ca, and all micronutrients (Cu, Zn, Fe, B, Mn, Mo); Ca was supplied in the superphosphate. Each plot had 8 x 8 trees, of which the 28 trees in the outermost rows of all plots were used for this experiment. The inner 6 x 6 trees were all pruned to 1.5 m in December 2010. The thinned stems were removed from the site but all other pruned material was left on the ground in the plantation.

3.2.2 Experimental treatments

Just before application of the thinning and fertiliser treatments, ten of the 28 trees with good form were selected from each plot giving a total of 180 trees for the 18 plots. Five of the ten selected trees were randomly allocated for pruning up to 1.5 m while the remaining five trees were left unpruned. All branches pruned were alive.

Immediately after pruning in December 2010, tree diameter at breast height (*dbh* at 1.3 m height) was 7.8 ± 0.2 and 7.9 ± 0.1 cm for unpruned and pruned trees, respectively. In the stem section below 1.5 m height in the 90 trees in each treatment, there were 8.4 ± 0.4 branches in the unpruned and 9.0 ± 0.3 stubs in the pruned trees. The majority of branches or pruning stubs were <2.0 cm diameter (d_b). The number of large branches (d_b >2 cm) in unpruned trees was 0.9 ± 0.1 and pruning wounds (d_b >2 cm) in pruned trees was 0.7 ± 0.1. These variables were not significantly different among the five unpruned or pruned trees in each plot (p >0.05). The number of older, unoccluded wounds that were present prior to setting up the trial was 1.9 ± 0.2 and 1.8 ± 0.2 in unpruned and pruned trees, respectively; this variable was not significantly different among the ten trees in each plot (p >0.05).

Assessments of the incidence and severity of stem discoloration and decay were conducted 18 months later in July 2012. Discoloration and decay severity was categorised into five ratings (Figure 3-1) modified from Barry et al. (2004). The ratings were roughly based on current industry guidelines where logs falling below nominal threshold values of 20 and 10% for discoloration and decay, respectively, can be considered acceptable for solid-wood products.

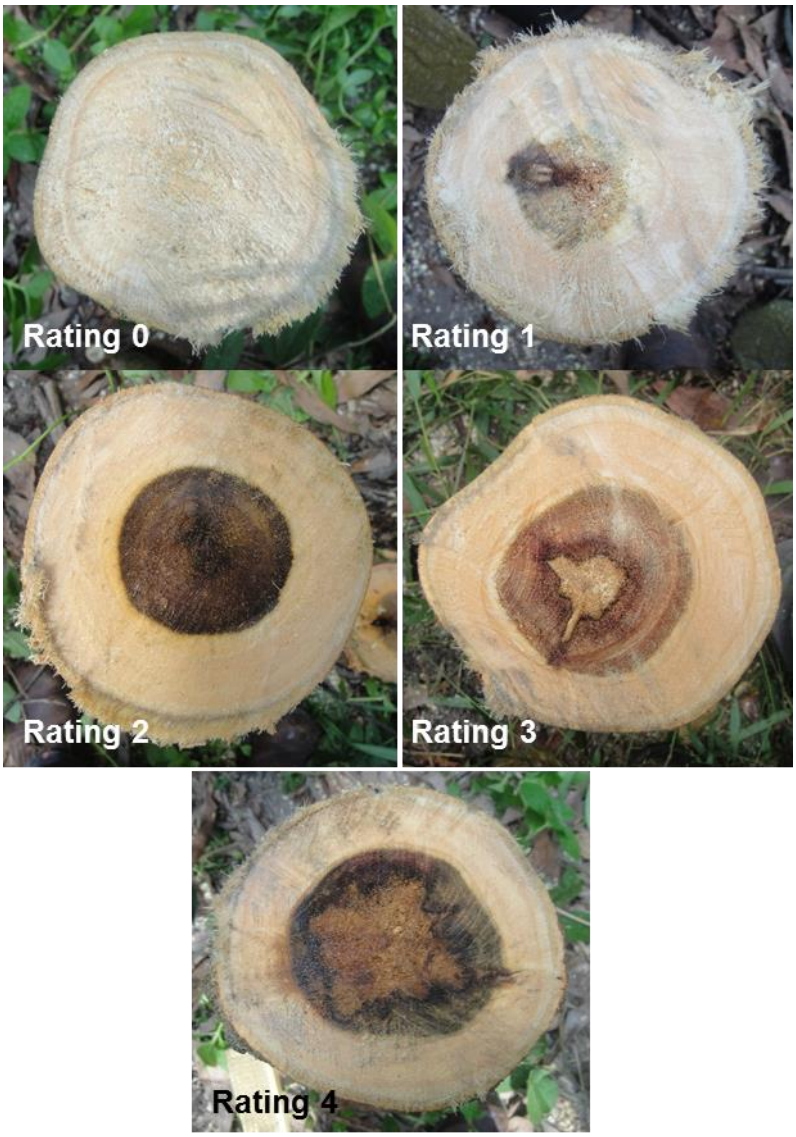

Figure 3-1: Examples of logs assigned to five discolouration and decay ratings; Rating 0; healthy wood i.e. no visible decay or discolouration, Rating 1; discolouration covering < 20% of the surface area, Rating 2; discolouration covering > 20% of the surface area, Rating 3; any discolouration plus decay covering < 10% of the surface area, Rating 4; any discolouration plus decay covering > 10% of the surface area.

Two trees in each of the unthinned and unpruned treatment were missing, presumably having died much earlier, and one tree in the thinned and unpruned treatment was dead. Each of the remaining 177 trees were felled and sectioned into three 0.5 m logs up to 1.5 m. The log with the most severe discoloration and decay was chosen for assessment. This was done by taking a photograph of the top-end of the selected log through a grid of transparent paper divided into 1.0 cm squares.

Log diameters, d_l (cm) were calculated by counting grid lines to an accuracy of 0.1 cm; log surface area (cm^2) = $\pi d_l^2/4$, where $\pi = 3.1414$; discoloration area (%) = (discoloration area/log surface area) × 100%; decay area (%) = (decay area/log surface area) × 100%. Discoloration and decay areas (cm^2) were measured using ImageJ 1.46r software (Schneider et al. 2012). Healthy area (%) = (healthy area/log surface area) where healthy area (cm^2) = log surface area – (discoloration + decay area).

For each plot, two pruned and two unpruned trees (total four trees $plot^{-1}$ x 18 plots = 72 trees) were randomly chosen from the 10 trees selected to examine responses to treatment between 1.5 m and 5.0 m height. Immediately after pruning in December 2010, the number of branches that had split away from the main stem because of wind damage was 0.11 ± 0.05 and 0.08 ± 0.05, and the number of large stem wounds 0.6 ± 0.11 and 1.0 ± 0.17, for unpruned and pruned trees, respectively. These variables were not significantly different among the two unpruned or pruned trees in each plot (p >0.05).

A total of 69 trees, excluding the three missing trees, were further sectioned in July 2012 into 0.5 m logs between 1.5 m and 5 m. The log with the most severe discoloration and decay was assessed using the same methods as above.

3.2.3 Data analyses

Differences between variable means were tested using SAS® Enterprise Guide® 6.1. © SAS Institute Inc. Cary NC USA. The means of variables below 1.5 m tree height of all 177 trees harvested were used to compare differences between treatments to test hypotheses (i), (ii) and (iii). The means of variables above 1.5 m tree height of the subset of 69 trees were used to test hypothesis (iv). Three-way analysis of variance was used to test for the main effects and interactions of fertiliser, thinning and pruning on all wood quality variables. The assumptions of ANOVA such as homogeneity of variance and the Gaussian distribution were evaluated by the use of quantile–quantile plots and residual plots for all variables. Fisher's protected least significant difference post hoc tests were used to determine significant differences (P=0.05) among treatment means. There were no significant effects of fertiliser on wood quality variables, therefore the results of fertiliser and their interactions are not presented.

3.3 Results

3.3.1 Incidence of discoloration and decay below 1.5 m tree height associated with pruning and thinning and their interaction

The incidence of discoloration and decay in the pruned treatment was significantly higher than in the unpruned treatment ($p < .001$) (Figure 3-2a); >80% and around 30% of trees, respectively, had discoloration and decay ratings of 3 and 4. No trees had a rating of 0 and 1 in the pruned treatment; there were 36% in the unpruned treatment.

The incidence of discoloration and decay was significantly higher in thinned than unthinned treatments ($p < .05$) (Figure 3-2b). Approximately 24% of trees had ratings of 0 and 1 in the unthinned and about 12% in the thinned treatment. Unthinned and thinned trees had a combined rating of 2 and 3 of 70 and 80%, respectively.

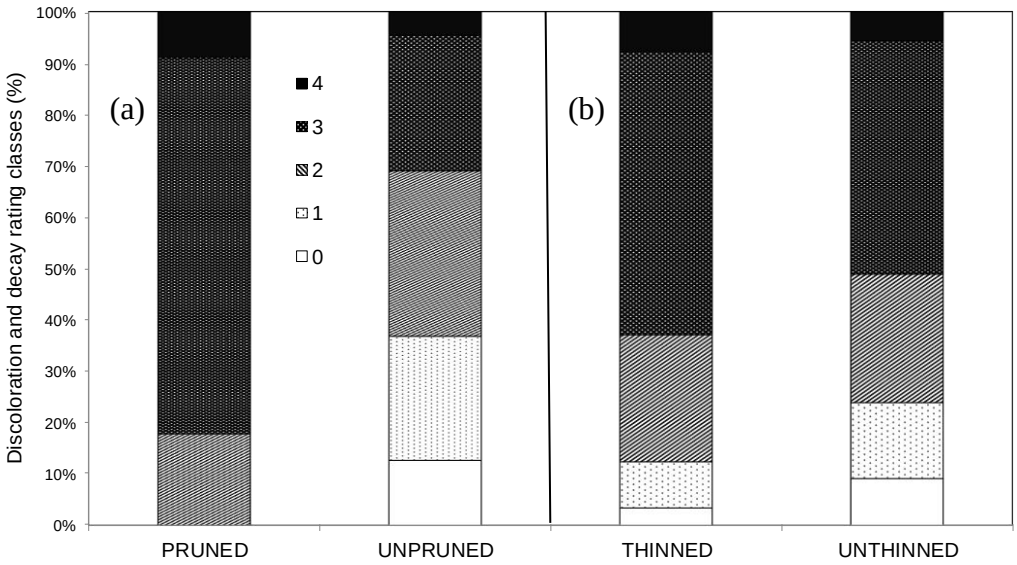

Figure 3-2: Percentage of trees in each discolouration and decay rating class (0 - 4) below 1.5 m tree height in (a) pruned and (b) thinned treatments for 177 trees 18 months after pruning and thinning.

3.3.2 Severity of discoloration and decay below 1.5 m tree height associated with pruning and thinning and their interaction

The percentage of decay area was significantly greater in pruned than in unpruned trees. Pruned trees had a mean rating of 2.9, which was significantly higher than the 1.9 for unpruned trees (Table 3-1a).

Table 3-1: Wood quality variables measured (a) below 1.5 m tree height for 177 trees and (b) above 1.5 m tree height for 69 trees in July 2012, 18 months after the pruning treatments.

Response variables	Pruned	Unpruned	Sig.
(a)			
Sample size (trees)	90	87	
Discoloration and decay rating	2.9 ± 0.1^{a}	1.9 ± 0.1^{b}	***
Decay area (%)	4.1 ± 0.4^{a}	1.80 ± 0.4^{b}	***
(b)			
Sample size (trees)	36	33	
Healthy area (%)	73.3 ± 1.4^{a}	87.1 ± 1.2^{b}	***
Discoloration and decay rating	2.7 ± 0.1^{a}	1.8 ± 0.1^{b}	***
Discoloration area (%)	21.4 ± 1.6^{a}	12.3 ± 1.2^{b}	***
Decay area (%)	4.6 ± 0.9^{a}	0.6 ± 0.2^{b}	***

Means with the same letter (a, b) are not significantly different (*** $p < .001$). Entries are means ± 1 SE.

The percentage of decay area was similar between thinning treatments (thinned: 3.3 ± 1.2% (mean ± 1 SE) and unthinned: 2.7 ± 1.3%, $p > 0.5$). The mean discoloration and decay rating was significantly less in the unthinned (2.2 ± 0.1) than thinned (2.6 ± 0.1) treatments ($p < .01$).

There was a significant pruning and thinning interaction for percentage of healthy wood and discoloration area but not for the discoloration and decay rating and the percentage of decay area (Table 3-2). Specifically, the percentage area of healthy wood was significantly smaller while those of discoloration was significantly greater in pruned than in unpruned trees within each thinning treatment. Thinning significantly increased the percentage discoloured area (and concomitantly decreased the percentage healthy area) for unpruned trees only. By contrast, percentage of healthy wood and discoloration area was similar, regardless of thinning treatment for pruned trees only.

Table 3-2: Wood quality variables measured below 1.5 m tree height for 177 trees in July 2012, 18 months after thinning and pruning treatments.

Response variables	Thinned		Unthinned		Sig.
	Pruned	Unpruned	Pruned	Unpruned	
Sample size (trees)	45	44	45	43	
Healthy area (%)	70.7 ± 1.4[a]	83.7 ± 1.6[b]	68.1 ± 1.0[a]	90.7 ± 1.1[c]	***
Discoloration area (%)	25.1 ± 1.3[a]	14.0 ± 1.5[b]	27.2 ± 1.2[a]	7.9± 0.9[c]	**

Means with the same letter (a, b, c) are not significantly different (** $p < .01$; *** $p < .001$). Entries are means ± 1 SE.

3.3.3 Severity of discoloration and decay above 1.5 m tree height associated with pruning and thinning and their interaction

All wood quality variables were unaffected by thinning and thinning x pruning interaction (data not shown). However, discoloration and decay rating, and percentages of discoloration and decay areas at >1.5 m height were significantly greater (and consequently decreased the percentage healthy area) in pruned than unpruned trees ($p < .001$) (Table 3-1b). The values for each variable were similar to those found at <1.5 m height.

3.4 Discussion

This study has shown that the incidence and severity of discoloration and decay in an intensively managed 3-year-old *Acacia* hybrid plantation was greater in treatments that had been pruned and thinned, 18 months after imposition of the treatments. The percentage healthy area was >68% and the percentage decay area was <5% across all treatment combinations. The importance of these results is now discussed in the context of the causal links between silvicultural pruning and thinning and discoloration and decay incidence and severity, and how this might affect the suitability of logs for solid-wood products.

Pruning significantly increased the incidence and severity of decay. This occurred in spite of pruning being undertaken in the dry season when penetration of decay fungi through pruning wounds is expected to be lower than in the wet season which is

considered more conducive to fungal infection (Lee & Arentz 1997). To our knowledge, only one other study has attempted to quantify the effect of pruning on discoloration and decay in tropical plantation species. This study found that pruning 1.5-year-old *A. mangium* trees in southern Sumatra had no effect on decay incidence 18 months after pruning; decay was absent possibly because there was an insufficient source of fungi present due to the trial being on an ex-grassland site (Beadle et al. 2007). In a temperate environment, live – branch pruning resulted in a significantly higher frequency of decay infections in pruned than unpruned trees of *E. nitens* three years after treatment; in pruned trees only, high rates of fertiliser applied at age five years had also resulted in a significantly higher incidence of decay than in unpruned trees (Wiseman et al. 2006). In the current study, application of fertiliser at pruning and thinning had no effect on discoloration and decay in *Acacia* hybrid. Interestingly, these fertiliser treatments had no significant effect on tree growth on this highly productive site (Kieu Tuan Dat, pers. comm.).

Previous studies have shown that decay is positively correlated with increasing diameter (d_b) of green branches, particularly those with d_b >2 cm (Mohammed et al. 2000). In the current study, all trees in both treatments had, on average, less than one branch of this size at the start of the experiment, thus the majority of branches or pruning stubs were <2.0 cm diameter. This suggests that pruned branches of d_b <2 cm in *Acacia* hybrid are linked to a potential for decay infection, as has similarly been found for temperate eucalypts (Pinkard et al. 2004). During form pruning prior to this experiment, all branches with d_b >3 cm had 50% of the length of their branches removed. The unoccluded wounds may have been a potential source of

discoloration and decay, but given that these wounds were some distance from the stem and that the sizes of the wounds were similar across treatments, their contribution to the observed results was most likely not substantial.

Thinning significantly increased the incidence of discoloration and decay below 1.5 m height but not above 1.5 m however, thinning did not affect the severity of discoloration and decay both above and below 1.5 m height. Although every attempt is made to minimise the problem, wounding and the subsequent development of discoloration and decay is commonly associated with thinning in forestry (Hunt & Krueger 1962; White & Kile 1991) including in this study. Strong wind events had also occurred at Nghia Trung in the period between the application of treatments and sampling that resulted in branch breakage, which may have been more prevalent in the thinned than unthinned stands (Pham Van Bon; pers. communication). Wounds from branch breakage can also lead to the development of decay, which potentially may explain the presence of discoloration and decay in the unthinned and unpruned trees in this study, although in an *A. mangium* stand in Malaysia, this was estimated to be the source of <3% of the observed heart rot (Lee 2002) and 7% in a thinned *Eucalyptus globulus* Labill. stand in Tasmania (Gerrand et al. 1997). In a survey of heart rot in *A. mangium* plantations in Sabah, Malaysia, Mahmud et al. (1993) found that only 6 and 4% incidence of discoloration and decay, respectively, were related to thinning wounds in a 3-year-old stand. While more certainty is required as to the origin of wounds associated with thinning, given that thinning did not affect discoloration and decay above 1.5 m tree height, wind most likely did not contribute to incidence and the severity of discoloration and decay in this study. Given that trees

were sampled only once in the study, the lack of thinning effect on severity may not be surprising. A sampling period of 18 months may not have been sufficiently long enough to fully capture the impact of thinning on severity. In an *E. nitens* plantation, decay column length assessed 5.5 years after pruning was 2-3 fold longer than 1.0 year after pruning (Barry et al. 2005a).

There was a significant thinning and pruning interaction for discoloration but not decay. In particular, thinning increased discoloration in the unpruned treatment only suggesting that thinning alone may have been a source of discoloration in this treatment. However, as pruning significantly increased the level of discoloration in both thinned and unthinned treatments, discoloration appears to have been mainly caused by pruning. Thus greater attention to pruning practice is required to minimise the development of discoloration in *Acacia* hybrid. Interaction of pruning and thinning on discoloration has not been examined previously, and as these treatments were carried out simultaneously, creating certainty about the individual effect of each on discoloration requires further investigation.

Pruning branches below 1.5 m tree height significantly increased the severity of decay and discoloration between 1.5 and 5 m tree height, even though no pruning was undertaken at 1.5 m tree height. This suggests that discoloration and decay had spread from the pruned into unpruned parts of the trees, or that existing discoloration and decay in the unpruned section had spread more rapidly after the pruning treatment (Wardlaw 2003). It has been suggested that decay columns associated with pruning can spread up to 2.5 m into unpruned sections in *E. nitens* (Barry et al.

2005a). In species that are resistant to decay fungi, the pattern of spread of discoloration and decay has been related to the ability of the host trees to compartmentalise the fungal pathogen (Shigo & Marx 1977; Shigo 1984). Thus it appears that *Acacia* hybrid is unable to prevent the spread of causal agents of heart rot. These results suggest that the susceptibility of *Acacia* hybrid to heart rot is similar to its female parent, *A. mangium* rather than its male parent, *A. auriculiformis* (Mahmud et al. 1993; Barry et al. 2002b; Bougher & Tommerup 2002; Ito 2002).

A five-category rating system was used to quantify the incidence and severity of discoloration and decay. A review of the relevant literature indicates a lack of consistency in such systems. Barry et al. (2004) also used a five-category rating and Mahmud et al. (1993) a six-category system, but both were qualitative and based on rough visual estimations of discoloration and decay. Wardlaw & Neilsen (1999) assessed decay in 12- to 16-year-old *E. nitens* using a rating system that did not differentiate between decayed and discoloured wood. Wardlaw et al. (2003) quantified decay percentage as stem volume where severe decay was ≥5% and 2 to <5% was moderate in intensively-managed temperate eucalypt forest of 22- to 34-year-old trees. These different approaches highlight the need to develop a consistent rating system for quantifying the impacts of management practices on the development of discoloration and decay, and their interrelatedness. Thus discoloration is not necessary associated with decay fungi (Wardlaw 1996) but rather the production of host chemical responses that change wood colour (Eyles et al. 2003a).

Our study found that 18 months after thinning and pruning of 3-year-old *Acacia* hybrid plantation trees, the incidence of discoloration and decay with a rating of 3 and 4 increased by 50% with pruning (below 1.5 m tree height). The severity of discoloration and decay increased by up to 25% (above 1.5 m tree height) and 5% (both above and below 1.5 m tree height), respectively with pruning. According to Nguyen Son (pers. comm.), a decay of <5% is not considered severe in Vietnam. However, given that *Acacia* hybrid plantations managed for sawlog production are typically harvested at age 7-8 years (Beadle et al. 2013b), then there is potentially time for the further development of discoloration and decay. Although the further development of discoloration will not exclude *Acacia* hybrid wood from solid-timber markets in Vietnam (Tran Thanh Cao, pers. comm.), further monitoring and changes to pruning and thinning practices may be required if decay levels are to be maintained within acceptable limits.

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Chapter 4

Wood-rotting basidiomycetes are a minor component of fungal communities associated with *Acacia* hybrid trees grown for sawlogs in South Vietnam

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Summary

Acacia hybrid (*Acacia mangium* × *A. auriculiformis*) clones are widely planted in Vietnam with a total of approximately 400,000 ha to meet the demand for pulpwood, sawn timber and wood chip exports. Silvicultural techniques such as pruning and thinning have been applied to improve productivity and sawlog quality of *Acacia* hybrid plantations. However, those techniques may also create opportunities for wood decay fungi to enter the *Acacia* hybrid stems through wounds and cause stem defects that reduce sawlog quality and the value of the plantation. The presence of fungal decay agents in *Acacia* hybrid trees was examined in two Vietnamese plantations. In July 2011, just prior to a second thinning, discoloured wood samples were taken from a three-year old *Acacia* hybrid plantation at Phan Truong Hai for the isolation of fungi. In July 2012, approximately 18 months after pruning and thinning treatments, discoloured wood samples were taken from a three-year old *Acacia* hybrid plantation at Nghia Trung for the isolation of fungi. DNA sequencing of the rDNA ITS identified the isolates. In May 2015, approximately four years after thinning and fertilizer treatments, discoloured and decayed wood samples were taken from the above (seven-year old) *Acacia* hybrid plantation at Phan Truong Hai for fungal identification. DNA was extracted directly from discoloured and decayed wood samples and fungal rDNA ITS amplicons sequenced on a Roche 454 sequencer. The results showed that silvicultural treatments did not affect the fungal communities associated with discoloured and decayed wood of *Acacia* hybrid plantation at Phan Truong Hai. A total of 135 fungal species or OTUs were identified, including 82 members of Ascomycota and 52 Basidiomycota.

4.1 Introduction

Acacia hybrid (*Acacia mangium* x *A. auriculiformis*) is widely planted in Vietnam with a total of approximately 400,000 ha (Nambiar & Harwood 2014). A significant proportion of the *Acacia* hybrid estate is being managed for solid wood production (Kha et al. 2012). Silvicultural techniques have been applied to increase the productivity and profitability of *Acacia* hybrid plantations in Vietnam (Gerrand et al. 1997; Wardlaw 2003; Medhurst et al. 2011) (Beadle et al. 2013a). However, both pruning and thinning can create infection courts for fungi (Medhurst et al. 2002; Pinkard et al. 2004) and thus lead to the risk of stem defect (Han & Kellogg 2000; Barry 2002). An investigation by Trang et al. (2017) showed that pruning and thinning increased the incidence and severity of discolouration and decay in a three-year old pruned and thinned *Acacia* hybrid plantation in Vietnam. An understanding of fungal agents associated with *Acacia* hybrid stem defect will contribute to successful management of *Acacia* hybrid plantations. Decay fungi can exist in wood of living trees at a low inoculum level, held in stasis by the high water content of sapwood (Chapela & Boddy 1988; Parfitt et al. 2010) and inhibited from growth in heartwood by secondary compounds (Malik et al. 2016; Mounguengui et al. 2016; Stirling et al. 2016). However, fungi still need a mode of entry, usually considered to be via wounds, either natural or man-made as in management operations such as pruning and singling. The incidence of heart-rot has been clearly linked to wound incidence (Hennon & DeMars 1997; Tonini & Halfeld-Vieira 2006).

A number of basidiomycete fungi have been associated with reduced productivity and profitability of *Acacia* hardwood plantations. For example, *Ganoderma applanatum* (Pers.) Pat. caused heart rot in *A. dealbata* in Australia and New Zealand (Bakshi 1976) and in *A. auriculiformis* in Australia (Old et al. 1996). *Phellinus noxius* (Corner) G. Cunn. caused lower stem rot and root rot of woody plants through Pacific to Southeast Asia region and its hosts include *Myristica fatua, Dysoxylum samoense, Hibiscus tiliaceus* (Brooks 2002), *Eucalyptus pellita* (Agustini et al. 2014) and *A. mangium* (Glen et al. 2014). *Phellinus badius* caused heart rot in *A. catechu* in India (Bakshi 1957).

The occurrence of heart rot in acacias in South-East Asia was noted by Gibson (1981) and has been the subject of several studies in Malaysia (Lee 2003). An unknown species of Polyporaceae was isolated from and implicated as the cause of heartrot in *A. mangium* (Hashim et al. (1990). *Phellinus noxius* was identified as the cause of a honeycomb rot that was only found in 7- and 8-year old *A. mangium* trees. The fungi isolated from the white fibrous rot that was more prevalent in younger trees were more diverse (Lee & Maziah 1993). Subsequently, Lee & Noraini Sikin (1999) reported that induction of sporocarps from cultures had enabled identification of *Rigidoporus hypobrunneus* (Petch) Corner, *P. noxius, Tinctoporellus epimiltinus* (Berk. & Broome) Ryvarden and *Oxyporus* cf. *latemarginatus* from *A. mangium* heart rot in Peninsular Malaysia and Kalimantan. In Vietnam, *A. mangium* plantations have been affected by heart rot caused by *Ganoderma* sp. (Thu 2016b).

Ascomycete fungi, particularly Xylariaceae, have also been associated with *Acacia* plantations (Sutherland & Crawford 1981; Nghi et al. 2012; Nnagadesi & Arya 2015). For instance, 16 species of Ascomycota were isolated from the discoloured and decayed wood of *A. mangium* in Malaysia (Lee et al. 1988). Most were considered to represent cosmopolitan, opportunistic wound invaders and common moulds, but the frequent isolation of a *Phialophora* sp. from discoloured wood and a sterile hymenomycete from the rotten wood prompted the question of whether the discolouration progresses to rot by the activity of a single fungal species, or whether invasion by pioneer fungi paves the way for a succession of more vigorous rotters. Subsequently, Lee & Maziah (1993) showed that *Lasiodiplodia* (*Botryodiplodia) theobromae*, *Fusarium*, *Gliocladium*, *Trichoderma*, *Cephalosporium* and *Phomopsis* were obtained from heart rot of *A. mangium* in Peninsular Malaysia. Some of them such as *Lasiodiplodia* are known to cause discolouration of wood. More recently, *Lasiodiplodia theobromae* was shown to incite the development of rot in *A. mangium* in Brazil (Halfeld-Vieira et al. 2006).

Significant advances have been made in assessing biodiversity of microbial communities in recent years by the application of metagenomics based on next generation sequencing technologies and DNA barcoding (Porter & Hajibabaei 2018). Quantifying fungal abundance based on sequence reads from rDNA amplicons will not give exact figures because of primer bias and variation in copy number among fungal species (Taylor et al. 2016) as well as additional factors that also affect other sampling methods such as patchy distribution and sampling effort. Studies have shown, however, that relative species abundances determined by amplicon

sequencing has high correlation to the actual relative abundances of artificial communities, though primer selection is critical to avoid amplification bias (Taylor et al. 2016). The primers used in this study amplify DNA from all fungal classes efficiently and span both internal transcribed spacer (ITS) regions of the rDNA (Gardes & Bruns 1993).

As there are no studies of the fungi associated with heart rot in *Acacia* hybrids in Vietnam, especially in pruned and thinned *Acacia* hybrid plantations which were managed for sawlogs, we used 454 sequencing in combination with fungal isolations to characterise fungal communities associated with discolouration and decay in Vietnamese *Acacia* plantations. The aim of this paper is to identify the fungi associated with discolouration and decay of *Acacia* hybrid plantations managed for sawlogs; to determine whether the fungi associated with advanced rot are the same as those associated with discoloured wood (incipient rot) and consider the risk they may pose to wood quality.

4.2 Materials and methods

4.2.1 Site and experiment design

Both sites are located in southern Vietnam, where the climate is characterized by distinct dry and wet seasons, the latter receiving >90% of the total annual rainfall of 1,500–2,500 mm from May to November; the mean annual temperature is 27.6–28.6°C with little monthly variation. The *Acacia* hybrid plantation at Phan Truong Hai was established on ferralsol soil in mid- August 2008. The site was planted at a

density of 1143 trees/ha (2.5 by 3.5 m spacing) and divided into 54 plots. A buffer row of trees around the four sides of each plot, resulted in plots (18.5 by 21 m) with 6 rows of 7 trees.

The *Acacia* hybrid stock consisted of a randomised mixture of four clones, TB01, TB06, TB11 and TB12 hybrids which had been developed from open-pollinated *A. mangium* female parents with the male parent being *A. auriculiformis*. The ramets were planted into 30 × 30 × 30 cm planting holes, with phosphorus fertiliser in the form of superphosphate equivalent to 50 kg P ha^{-1} placed in the bottom of the pit just prior to planting. Round-up at 4 L ha^{-1} (0.48 g L^{-1} and 1.92 kg ha^{-1} glyphosate isopropylamine salt) was applied with a shrouded sprayer on four occasions, once before planting, and at ages 2.5, 10 and 14 months. Fertiliser treatment after planting: 100 g $tree^{-1}$ of N:P:K at 16:16:8 (= 18.3 kg P ha^{-1}) plus 385 g $tree^{-1}$ superphosphate (= 31.7 kg P ha^{-1}) in a circle 40 cm from the tree applied in November 2008. Trees were singled in March 2009 (7 months after planting) and form pruned by removing approximately one-half of the length of competing leaders and branches using secateurs and pruning saws in September 2009 (13 months after planting, in the wet season). A second form pruning, to 4 m height, was conducted in January 2010 (17 months after planting, in the dry season).

The plots were arranged into three randomised complete blocks with 18 treatment combinations. Treatments comprised the following: three thinning densities, (1143, 600 and 450 stems ha^{-1}) ; two thinning times (approximately 2 and 3 years after planting); three additional fertiliser treatments at thinning (no fertiliser, 50 kg P ha^{-1}

and 50 kg P ha^{-1} + basal fertiliser). Only half of all plots (27 plots), i.e. those thinned at 3 years after planting, were investigated in this study. The basal fertiliser contained salts of all other macronutrients (K, Mg) except N and Ca, and all micronutrients (Cu, Zn, Fe, B, Mn, Mo); Ca was supplied in the superphosphate. At the time of sampling, average stem diameter at 1.3 m height was 17.12 cm (± 2 cm) and average tree height was 20.31 m (± 0.3 m). The cut stems were removed from the site but all other pruned material was left on the ground in the plantation.

Site and experimental design of the *Acacia* hybrid plantation at Nghia Trung has been described in Trang et al. (2017).

4.2.2 Tree sampling

Samples of discoloured wood (incipient decay) were taken from Phan Truong Hai in 2011 and from Nghia Trung in 2012 for fungal isolation. Isolations were made from wood with incipient decay to avoid contamination by secondary colonisers in wood with advanced decay. In a third sampling, wood samples with both incipient and advanced decay were collected from Phan Truong Hai in 2015 for fungal amplicon sequencing. A wood sample was scored as decayed if a loss of structure and/or density was visible, see Figure 1 in Trang et al. (2017). If it was discoloured but still hard when tapped with a chisel, it was scored as discoloured. Advanced decay was usually surrounded by a region of incipient decay that was evident by discolouration, so two samples were taken from logs with advanced decay but only one from logs with incipient decay.

In July 2011, just before the thinning and additional fertiliser treatments, three trees were randomly selected from each of the 27 plots at Phan Truong Hai. These 81 trees, at age 3 years, were felled and each tree sectioned into ten 0.5 m logs. Discoloured wood samples (approximately 1 cm wide x 1 cm high x 2 cm long, one per log) were systematically taken from the top-end of each log, and from the stump, and stored in paper bags until fungal isolation (within 2 months).

At Nghia Trung a total of 177 trees in a pruned, thinned and fertilised *Acacia* hybrid plantation were felled in July 2012 and sectioned up to a height of 1.5 or 5 m into three or ten 0.5 m logs [see experiment treatment details in Trang et al. (2017)]. Discoloured wood blocks at the top-end of discoloured logs were taken (approximately 15 mm wide x 15 mm deep x 25 mm long) and stored in paper bags until fungal isolation (within 2 months).

In May 2015, approximately four years after thinning and fertiliser treatments, 3 trees were randomly selected from each of the 27 plots previously sampled at Phan Truong Hai, felled and sectioned up to a height of 5 m into ten 0.5 m logs. Of the 810 logs, 172 logs had visible decay and/or discolouration as described above. Samples of discoloured and decayed wood were taken from the top-end of each of the 172 logs, air-dried under the sun and transported to the University of Tasmania in compliance with quarantine procedures under permit to import quarantine material IP14010539.

4.2.3 Fungal isolation

In 2011, isolations were attempted from discoloured wood samples collected from the *Acacia* hybrid plantation at Phan Truong Hai and in 2012, from discoloured wood samples collected from the *Acacia* hybrid plantation at Nghia Trung. The isolation process was as follows: each wood sample was sterilised by submerging in 95% ethanol for one minute, then in sodium hypochlorite (approximately 2.5% available chlorine) for two minutes and then in distilled and sterilised water for one minute and placed onto MAT medium (1% malt extract (Morgan's Brewing Co., Yatala, Queensland), 20 g agar, 950 ml water, 50 ppm penicillin, 50 ppm streptomycin, 25 ppm polymyxin and 230 ppm thiabendazole) at room temperature (20 - 25 ^{o}C) for 2-3 weeks. Wood samples from logs with black, radial streaks on the cut surface were treated as potentially infected by *Ceratocystis manginecans.* Such samples were incubated in moist Petri dishes and isolates obtained by carrot baiting as described by (van Wyk et al. 2009). Each isolate was subcultured on MA (1% malt extract, 20 g agar, 1000 ml water) at 20 – 28 ^{o}C. Two-week old colonised agar blocks (10mm diameter x 2mm deep) were stored in tubes containing sterile, distilled water at 4 ^{o}C.

4.2.4 Identification of fungal isolates by DNA sequencing

In 2013, the isolates obtained from Nghia Trung and Phan Truong Hai were subcultured onto malt extract agar (MA). Due to the long-time in storage, many isolates (50% and 65% respectively) were either contaminated or non-viable. The

DNA of isolates was extracted using the glassmilk method described by Glen et al. (2002) and transported to University of Tasmania in compliance with quarantine procedures under Permit to import quarantine material IP14010539. The ribosomal DNA internal transcribed spacers (rDNA ITS) 1 and 2, including 5.8S and a part of 18S and 28S regions of nuclear rDNA were amplified using the primer pair ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Amplification parameters were an initial denaturation at 95 ^{o}C for 3 minutes, followed by 35 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds and extension at 72 ^{o}C for 30 seconds, with a final extension at 72 ^{o}C for 7 minutes on a PTC 225 Peltier Thermal Cycler. Each set of PCR reactions included a positive (fungal isolate DNA) and a negative (no template DNA) control, amplification was checked by electrophoresis on agarose gels. Negative controls were consistently negative. The PCR products were sequenced by Macrogen Inc. in Korea using both forward primer ITS1-F and reverse primer ITS4. The obtained sequences were edited using Geneious version 7.1.9 (Biomatters Ltd.). The edited sequences were grouped into clusters at 95% similarity using CD-hit-est tool (Fu et al. 2012) for Nghia Trung and Phan Truong Hai, separately. The fungal operational taxonomic units (OTUs) represented by each cluster were identified by BLAST searches (Altschul et al. 1990) on NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine).

4.2.5 PCR and DNA sequencing directly from wood

DNA was extracted directly from discoloured and decayed wood samples collected from Phan Truong Hai (May 2015) approximately two months after collection. Briefly, the wood block was sampled by drilling through the discoloured or decayed areas with a 3.2 mm diameter drill bit. The drill bit was sterilised by dipping in 70% ethanol and flaming for 1 minute before each drilling. A clean piece of paper was placed on the worktable to collect drill shavings from each sample. Drill shavings (approximately 300 µl) and three tungsten carbide beads (3 mm diameter, Qiagen) were transferred to a PowerLyzer Glass Bead Tube (component of the PowerLyzer®PowerSoil® DNA Isolation Kit, MoBio). The tube was immersed in liquid nitrogen for 2 minutes and placed in a high-speed shaker (TissueLyser II - Qiagen) and shaken for 5 minutes with a frequency of 30 Hz. The DNA was purified using a PowerLyzer®PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc.), following the manufacturer's instructions. The final 100 µl of eluted DNA was stored at - 20ºC.

DNA was amplified using fusion primers, the forward primer included A-side primer for 454 sequencing, one of 12 multiplex identifier sequences (MIDs) and ITS1-F on the 3' end. The reverse primer consisted of the B-side primer plus ITS4. DNA from three trees in each of three plots with the same thinning and fertiliser treatments were amplified independently using the same MID on the forward primer. PCR reagents and amplification parameters were as described above. PCR products were purified

using UltraClean® PCR Clean-up Kit (MOBIO Laboratories, Inc.). The PCR clean-up processes were conducted following the manufacturer's instructions.

PCR products were analysed on the Fragment Analyzer Automated CE System using DNF-900 dsDNA Reagent Kit (DNF-910-K0500) (Advanced Analytical Technologies, Inc.). The PCR products (approx. 10 ng from each sample) were then combined into two pools, precipitated and resuspended in a final volume of 21 µl for each pool. The DNA concentrations were 16.18 and 13.59 µg/µL for pool 1 and pool 2, respectively.

The rDNA amplicons were sequenced on a Roche GS FLX 454 sequencer by Macrogen Inc. in Korea. Reads were trimmed for length (> 300 bp) and quality (bases with a quality score of 10 or less were removed from both ends of each read).After demultiplexing using CLC Genomics Workbench (Quiagen), reads without legible barcodes were removed and the remaining reads grouped into clusters at 95% similarity using CD-hit-est tool (Fu et al. 2012). The fungal operational taxonomic units (OTUs) represented by each cluster were identified by BLAST tool on NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine).

The OTUs of 454 sequencing were compared with the OTUs of isolates collected from Nghia Trung (2012) and Phan Truong Hai (2011) using "Map to reference" tool in Geneious version 7.1.9 (Biomatters Ltd.).

4.2.6 Statistical analysis

The number of reads per OTU in each sample were standardised by converting to a percentage of the total reads per sample. The relative abundance of fungal taxa identified by 454-sequencing method was based on the average percentage of reads per sample for each fungal taxon (Jumpponen & Jones 2009). The relative abundances of the sequence reads for the 50 most abundant OTUs were graphed in Microsoft Excel. Singletons were removed for calculation of species accumulation curves (Wen et al. 2017) with 95% confidence intervals using EstimateS version 9.1.0 (Colwell 2013) and also graphed in Microsoft Excel.

The scaled reads were then log transformed (log(x+1)) to reduce the effect of very large values prior to analysis of fungal communities using the program PRIMER v6 with PERMANOVA + (Anderson et al. 2008). Samples of discoloured and decayed wood, with three thinning treatments in combination with the three levels of fertilizer treatment, were subjected to an unconstrained ordination using nonmetric multidimensional scaling. Statistical tests were carried out with PERMANOVA and canonical analysis of principal co-ordinates (CAP) (Anderson & Willis 2003) using 9999 permutations to assess fungal community differences between discoloured and decayed wood samples, taking into account thinning and fertilizer treatments.

4.3 Results

4.3.1 Fungal isolations

From the discoloured wood samples, 191 fungal isolates were obtained from Phan Truong Hai and 325 from Nghia Trung. Only 67 and 164, respectively, of these were successfully recovered after storage.

4.3.2 Fungal isolates obtained from *Acacia* hybrid plantations at Nghia Trung and Phan Truong Hai

A total of 159 isolates obtained from Nghia Trung and 67 isolates obtained from Phan Truong Hai have been identified (Appendix 4-1). Of these, 111 isolates obtained from Nghia Trung and 55 isolates obtained from Phan Truong Hai were classified into 22 different Ascomycota taxa (Appendix 4-1). Another 42 isolates from Nghia Trung and 12 isolates from Phan Truong Hai were identified as 12 basidiomycete taxa (Appendix 4-1) and the remaining 6 isolates were identified as *Gongronella* sp. cf *butleri* belonging to Mucoromycota. Wood-rotting basidiomycetes constituted under 25% of the isolates in this study despite the use of antibiotics selective for Basidiomycota (Appendix 4-1).

Pseudopestalotiopsis sp. (29 isolates from Nghia Trung and 19 from Phan Truong Hai) and Nectriaceae sp. 2 (40 isolates from Nghia Trung) were the most frequently isolated fungal species. A total of 161 isolates belonged to the Sordariomycetes,

making this the most abundant fungal class, followed by Agaricomycetes (all 54 isolates of Basidiomycota) and Mucoromycota (6 isolates).

4.3.3 Direct DNA sequencing from wood samples – species abundance and diversity

A total of 124,471 reads were obtained from the two amplicon pools. After trimming for length and quality this was reduced to 8,067, with 117-1,091 reads per demultiplexed sample. The 8,067 reads were grouped at 95% similarity into 437 OTUs of which 155, over one third, were singletons. A total of 75 OTUs, constituting 85% of the trimmed reads, were identified (Appendix 4-2) by BLAST searches of the INSDs (International Nucleotide Sequence Databases). These 75 OTUs included only seven members of the Basidiomycota, so a database of the representative OTU sequences was searched for sequences with high similarity to 5.8S sequences from Basidiomycota. This indicated that another 42 of the 437 OTUs may also be members of the Basidiomycota. These OTUs were also identified by BLAST searches, 34 of them were confirmed as Basidiomycota, making a total of 41, and included in Appendix 4-2. Wood-decaying basidiomycetes represented a very small proportion of the fungal species detected in stems of living *Acacia* trees and were present in low abundance and at low frequency (Table 4-1).

Table 4-1: Potential wood decay basidiomycetes detected by 454 sequencing from Nghia Trung or isolated from Nghia Trung (NT) or Phan Truong Hai (PTH), in order of relative abundance of sequence reads (Sum of % reads in 9 pooled samples from

discoloured wood and 9 pooled samples from decayed wood) or number of isolates for species that were not detected by environmental sequencing.

Species	Discoloured wood		Decayed wood		Number of isolates		Genbank accession numbers
	Number of samples	% of reads	Number of samples	% of reads	NT	PTH	
Phellinus sp. cf. *resupinatus*	2	1.47	3	4.93			MF942544
Pseudolagarobasidium acaciicola	0	0.00	2	3.76	2	2	KY990563
Ganoderma sp. 1	1	2.61	0	0.00			MF942545
Trametes versicolor	3	1.75	1	0.56			MF942555
Cabalodontia sp.	1	1.40	0	0.00			MF942549
Stereum sp.	1	0.84	0	0.00			MF942558
Agaricomycetes sp. 1	2	0.76	0	0.00			MF942562
Coniophora sp.	1	0.75	0	0.00			MF942540
Lentinaceae sp.	1	0.75	1	0.00			MF942548
Sistotrema sp.	0	0.00	1	0.54			MF942541
Irpicaceae sp.	1	0.47	0	0.00			MF942556
Polyporaceae sp. 4	1	0.45	0	0.00			MF942553
Meruliaceae sp.	1	0.43	0	0.00			MF942550
Phanerochaetaceae sp.	1	0.42	0	0.00			MF942561
Corticiaceae sp.	0	0.00	1	0.42			MF942542
Agaricales sp. 1	0	0.00	1	0.42			MF942560
Psathyrellaceae sp. 2	0	0.38	1	0.00			MF942538
Peniophora sp.	1	0.37	0	0.00			MF942557
Ganoderma sp. 3	1	0.36	0	0.00			MF942547
Ganoderma sp. 2	1	0.12	0	0.18			MF942546
Agaricales sp. 2	0	0.00	1	0.29			MF942539

Species	Discoloured wood		Decayed wood		Number of isolates		Genbank accession numbers
	Number of samples	% of reads	Number of samples	% of reads	NT	PTH	
Junghuhnia sp.	0	0.00	1	0.27			MF942551
Polyporaceae sp. 3	1	0.24	0	0.00			MF942554
Pseudotomentella larsenii	1	0.24	0	0.00			MF942559
Cerrena sp.					12		MF033437
Polyporaceae sp. 2					9	1	MF621974
Schizophyllum commune					3	6	KY930913
Polyporales sp.					5	1	MF621967
Peniophora cf. *lycii*					4		MF621975
Polyporaceae sp.1					3		MF621972
Psathyrellaceae sp. 1						2	MF621966
Phellinus noxius					1		MF621969
Phlebiopsis flavidoalba					1		MF621970
Phlebiopsis sp.					1		MF621971
Trametes aff. *cubensis*					1		MF621973

4.3.4 Species diversity and relative abundance as determined by DNA sequence data

A total of 109 fungal taxa have been identified by DNA extracted directly from discoloured and decayed wood samples (Appendix 4-2), the remaining 328 OTUs, all with fewer than 10 reads per OTU, have not been identified. Within the 109 identified OTUs, the greatest diversity was in the Dothideomycetes class with 35 taxa, followed by Agaricomycetes and Sordariomycetes, each with 24 taxa and

Tremellomycetes with 12 taxa. The species abundance curve (Figure 4-1) shows a few very abundant taxa with a steep drop-off in abundance and a large number of rare species. Only one wood-rotting basidiomycete, *Phellinus* sp., was among the 50 most abundant taxa (Figure 4-1). The twenty most abundant OTUs include ten unidentified members of the Xylariales. Of these, five were present in all or all except one of the 18 pooled samples (Table 4-2). The species accumulation curve, omitting singletons, (Figure 4-2) is predicted to reach an asymptote between 350 and 400 OTUs, indicating that further sampling would be unlikely to detect many additional fungal OTUs in discoloured or decayed wood of living *Acacia* hybrid trees at Phan Truong Hai.

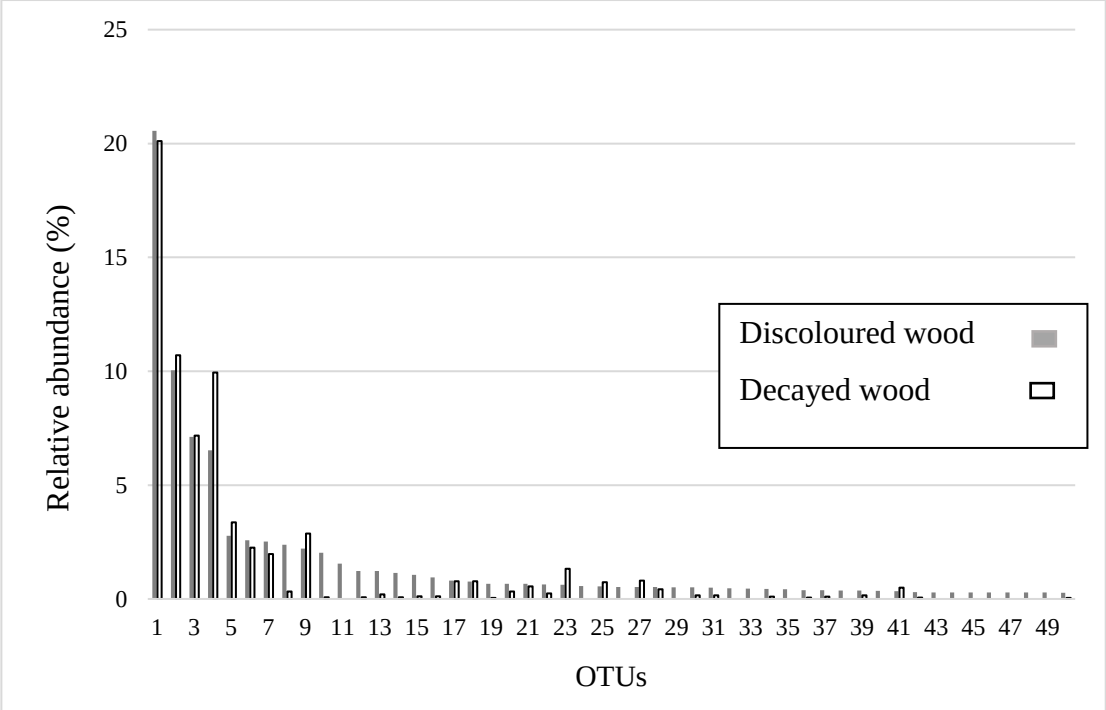

Figure 4-1: Relative abundance (% reads per sample) of 50 OTUs in discoloured and decayed wood. The only wood decay basidiomycete in the top 50 OTUs (by abundance) is *Phellinus* sp. (OTU 39).

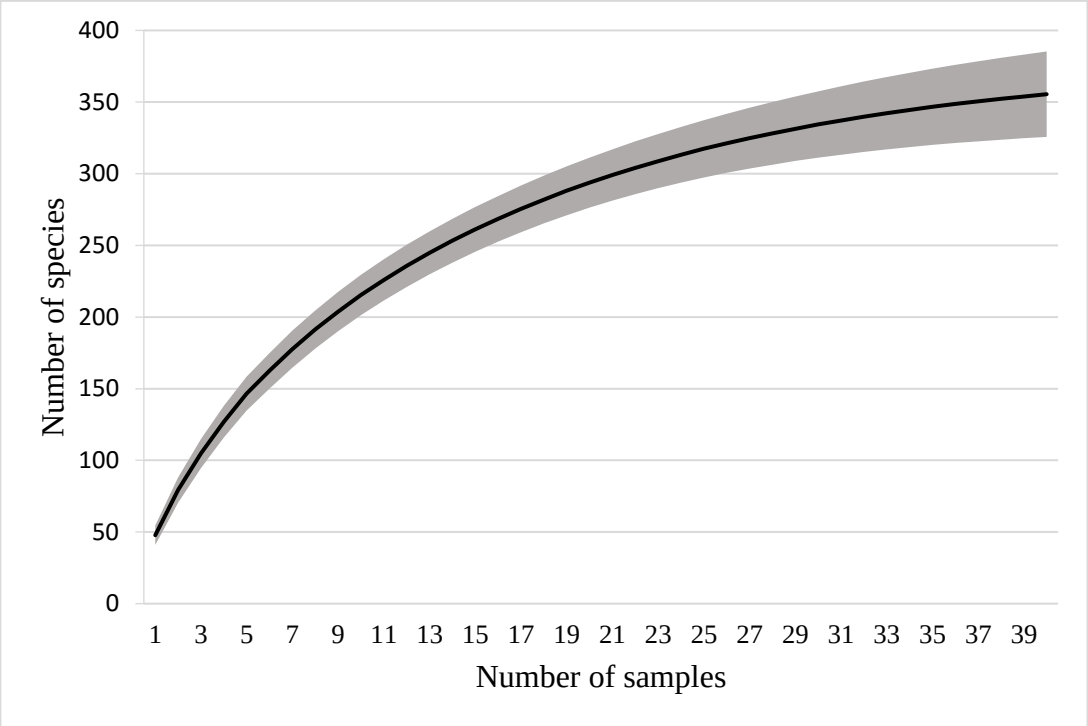

Figure 4-2: Species accumulation curve for OTUs identified by 454-sequencing directly from discoloured and rotten *Acacia* wood, extrapolated to 40 samples with 95% confidence limits shaded in grey.

Table 4-2: Relative abundance, expressed as mean read % per sample, of the 20 OTUs with highest abundance across all 18 pooled samples, in descending order.

Species	Number of samples		Mean % abundance	Genbank accession numbers
	Discoloured	Decayed		
Xylariales sp. 1	9	9	20.3	MF942527
Cladosporium sp. 1	8	9	10.4	MF942472
Xylariales sp. 2	8	9	8.2	MF942530
Xylariales sp. 3	9	9	7.1	MF942531
Nigrospora sp.	8	8	3.1	MF942524
Xylariales sp. 5	9	9	2.5	MF942533
Xylariales sp. 4	9	9	2.4	MF942532
Dothideomycetes sp. 2	5	7	2.2	MF942504
Pyrenochaetopsis sp. 2	0	3	1.4	MF942499
Xylariales sp. 11	1	1	1.3	MF942529
Leptoxyphium sp.	3	2	1.1	MF942471
Xylariales sp. 6	3	3	1.0	MF942534
Xylariales sp. 9	0	2	0.8	MF942537
Trichoderma sp. 1	0	1	0.8	MF942518
Neopestalotiopsis sp.	5	7	0.8	MF942525
Capnodiales sp. 5	2	1	0.8	MF942488
Pyrenochaetopsis sp. 1	3	5	0.8	MF942498
Xylariales sp. 8	3	3	0.7	MF942536
Xylariales sp. 10	5	7	0.7	MF942528
Pseudopithomyces chartarum	2	2	0.7	MF942497

4.3.5 Statistical analyses of fungal communities from discoloured and decayed wood

The thinning and fertiliser treatments and their interaction did not affect the fungal communities associated with discoloured and decayed wood of *Acacia* hybrid plantation at Phan Truong Hai ($p < .05$). There was also no significant difference between fungal communities associated with rotten wood and those associated with discoloured wood when tested by PERMANOVA ($p = 0.774$) or by CAP ($p = 0.745$).

Although the fungal communities in discoloured and decayed wood samples were not significantly different, 26 fungal OTUs (including 13 potential wood decay basidiomycetes) were only associated with discoloured wood samples and another 26 OTUs (7 potential wood decay basidiomycete fungi) were only associated with decayed wood samples. However, these OTUs mainly occurred at low frequency and low abundance (Appendix 4-3). Another 57 fungi were associated with both discoloured and decayed wood samples.

4.3.6 Comparison of fungal isolates and 454-sequence OTUs at the two sites

Of the 32 fungal species isolated from Nghia Trung and 15 from Phan Truong Hai, 11 were common to both sites (Appendix 4-1). Three of them, *Neopestalotiopsis* sp., *Pseudolagarobasidium acaciicola* and *Pseudopestalotiopsis* sp., were isolated from both sites and were detected by 454-sequencing method. Another eight species (*Ceratocystis manginecans*, *Clonostachys* sp. 1, *Colletotrichum* sp., *Fusarium* sp. 3,

*Pestalotiopsi*s sp. 2, Polyporaceae sp. 2, Polyporales sp.1 and *Schizophyllum commune*) were isolated from both sites and four species (*Fusarium* sp. 2, Nectriaceae sp. 1, *Trichoderma* sp. 1 and *Trichoderma* sp. 2), were isolated from Nghia Trung and detected by 454-sequencing at Phan Truong Hai. Additionally, two species (Botryosphaeriales sp. and *Debaryomyces* sp. cf *fabryi*) were isolated and detected by 454-sequencing method at Phan Truong Hai (Appendix 4-4). The remaining 420 OTUs were not detected by isolation, including the six most abundant OTUs, all members of the Xylariales.

4.4 Discussion

By all measures, species/OTU richness, NGS read abundance and fungal isolation, wood decay basidiomycetes were a minor component of the fungal communities in living Acacia hybrid trees in two Vietnamese plantations. We detected 35 wood decay species, 12 by isolation, 24 by environmental DNA sequence data and only one by both methods. By contrast, the number of ascomycete OTUs, excluding singletons, exceeded 250. The low overlap between fungi detected by isolation and those detected by environmental sequencing has been observed previously in wood (Jang et al. 2016) and other substrates (Arnold et al. 2007). It is unclear how much this is due to sampling bias, e.g. primer specificity, growth media, and how much to stochasticity.

As a group, wood decay basidiomycetes accounted for under 2% of the total 454 sequence reads, with each OTU detected in at most 5 of the 18 pooled DNA samples.

This is consistent with the theory that wood decay basidiomycetes are held in stasis in living wood by osmotic or chemical conditions (Chapela & Boddy 1988). By contrast, the most abundant OTU, an unidentified species of Xylariaceae, accounted for over 20% of the total reads and was present in all 18 DNA samples. Another four species of Xylariales were among the seven most abundant OTUs and were detected in all or all but one of the 18 DNA samples. None of these Xylariales species was isolated. The high abundance and almost ubiquitous presence of these species indicate that these fungi were not suppressed by conditions within the wood of living trees, but may be actively growing without damaging their host. The lack of isolation may have been due to the use of selective media, overgrowth by fast-growing fungi, or it may be that they are obligate endophytes. Rogers (2000) described nine groups of Xylariacious lifestyles including four that occur in stems of living plants. Two of these groups inhabit living material latently in a similar manner to the basidiomycete wood decay fungi, until conditions (death of host, host stress) are conducive to proliferation. A third group occupy 'dead islands' within living stems and the fourth are classed as true endophytes. The last group are the least well-known and a deeper understanding of their ecology will depend on molecular identification (Rogers 2000). The high prevalence and abundance of Xylariaceae spp. in *Acacia* stems invites further investigation to gain a deeper understanding of their roles and ecological functions.

The higher species diversity detected by amplicon sequencing compared to fungal isolations in this study was consistent with other studies in a range of substrates (Oono et al. 2015; Rocchi et al. 2017) and may reflect growth media requirements

(Muggia et al. 2017). The predominance of Ascomycota in stems of living trees is also consistent with other tree species. For example, a study of fungi associated with heart rot in *Alnus glutinosa* stands in Latvia by Arhipova et al. (2012) showed that among 1134 isolates from sound and decayed wood samples taken from living stems of *Alnus glutinosa* there were 47 ascomycete and 17 basidiomycete fungal taxa identified.

In this study, the predominant species isolated were Nectriaceae spp. including *Fusarium* spp., *Pseudopestalotiopsis* sp. and *Pestalotiopsis* spp. These genera are common in tropical forests and include several plant pathogens (Maharachchikumbura et al. 2014) but also many endophytes with unclear functions (Reddy et al. 2016). *Pestalotiopsis* spp. have been reported to cause disease on the leaves of *Acacia* spp. and *Eucalyptus* spp. plantations in Vietnam (Nghia 2010, 2015). However, the disease was of low severity and did not affect productivity of the plantations (Nghia 2010, 2015). In tropical areas, some *Pestalotiopsis* species such as *P. theae* cause serious disease on oil palm leaves, as reported in Thailand (Suwannarach et al. 2013), though Reddy et al. (2016) consider that many *Pestalotiopsis* species, including *P. theae,* are generalist endophytes in a range of tropical trees. *Pestalotiopsis* species were also among the most abundant litter degraders of tropical trees (Prakash et al. 2015) and these authors hypothesise that fungi adapted to a particular environment may evolve into a more generalist lifestyle, occupying living host tissue and also degrading litter. Endophytic species may become more aggressive and proliferate in stressed hosts, in which circumstance they may be assigned a pathogenic status (Steinrucken et al. 2017). Others may behave

similarly to the wood decay basidiomycetes, surviving at low levels until the death of the host, at which time those fungi already occupying the substrate gain first access to the available nutrients (Chapela & Boddy 1988).

Fusarium spp. have been reported to cause disease in forest trees, especially seedlings in the nursery. Coniferous trees including Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), larch (*Larix* spp.), true firs (*Abies* spp.) western white pine (*Pinus monticola* Doughl.) and sugar pine (*Pinus lambertiana* Dougl.) are affected by *Fusarium* spp. (Brownell & Schneider 1983; Enebak et al. 1990; James 2012). Gordon et al. (2015) reported that coniferous trees could suffer significant damage from seedling diseases caused by *Fusarium* spp. and from pitch canker caused by *F. circinatum* Nirenberg & O'Donnell. *Fusarium circinatum* is has spread to forests and nurseries in Spain, Portugal, France, Italy and South Africa (Wingfield et al. 2008; Bezos et al. 2017). *Fusarium oxysporium* f. sp. *koae*, a vascular wilt disease of *Acacia koa*, has caused high rates of mortality in field plantings and threatens native *A. koa* forests in Hawai'i (Dudley et al. 2015). In Vietnam, *Fusarium* spp. have also been reported to cause disease in forest trees including damping-off disease in *Pinus* seedlings caused by *F. oxysporum* Schltdl. (Nga 2015), and asparaginase disease caused by *F. equiseti* (Corda) Sacc. in *Dendrocalamus membranaceus* (Nga & Thu 2006). *Fusarium oxysporum*, *F. moniliforme* (syn. *F. fujikuroi* Nirenberg) and *F. euwallaceae* sp. are now reported to cause damping-off disease of *Acacia mangium*, *A. auriculiformis* and *Acacia* hybrid in the nursery (Nghia 2010, 2015). *Fusarium euwallaceae* is vectored by the ambrosia beetle *Euwallacea fornicatus*

Eichhoff in Vietnam and can infect *A. auriculiformis* and *A. mangium,* causing a vascular wilt that can kill the trees (Thu 2016a).

The canker and wilt pathogen, *Ceratocystis manginecans,* was isolated from both sites, though the sampling strategy targetted wood-rot fungi rather than *Ceratocystis*. For this same reason, tree deaths due to *Ceratocystis* were not examined in this study. The relatively low level of *Ceratocystis* isolation may mask its true prevalence at these sites. Three of the *C. manginecans* isolates from this study were pathogenic when tested against nine *Acacia* genotypes in a recent study (Trang et al. 2018) and were more aggressive than three basidiomycete isolates (Trang 2017, unpublished). *Ceratocystis manginecans* has been reported to cause severe disease in *Acacia* species, especially *A. mangium* in Indonesia, Vietnam and Malaysia (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015). Disease caused by *C. manginecans* is a major constraint to productivity of *A. mangium* (Brawner et al. 2015) in SE Asia but *A. auriculiformis* and some of its hybrids with *A. mangium* appear to have higher potential for disease resistance or tolerance (Trang et al. 2018). In Vietnam, the incidence of disease caused by *C. manginecans* in plantations of *A. auriculiformis*, *A. mangium* and *Acaci*a hybrid plantations in Vietnam ranges from 7.1-12.5%, 9.2-18.4% and 10.2-18.2%, respectively (Thu et al. 2016). Though detected in only a small proportion of the trees sampled in this study, rapid build-up of inoculum levels and hence disease have been demonstrated in Malaysian plantations of the highly susceptible *A. mangium,* leading to deployment of alternative species in pulpwood plantations (Brawner et al. 2015). Screening of clones for resistance/tolerance to

C. manginecans before commercial deployment represents the best strategy to reduce the risk of a similar fate for Vietnamese Acacia plantations.

Some of the identified isolates have the potential to cause heart rot. These include *Phellinus noxius*, which has been reported to cause root rot in trees such as *Sterculia foetida* in Taiwan (Lin et al. 2013). This species has been isolated from *Khaya senegalensis (*Desr.) A. Juss pruning wounds in Singapore and caused extensive wood decay after inoculation into living *K. senegalensis* (Burcham et al. 2015). This fungus has also been reported to cause brown root-rot disease on *A. mangium* in the Philippines, Malaysia, Indonesia and India (Mehrotra et al. 1996; Militante & Manalo 1999; Lee 2000; Irianto et al. 2006). In Vietnam, a related species, *P. pachyphloeus* has been reported to cause heart rot in Acacia hybrid and *A. mangium* plantations with low severity (Nghia 2006, 2010).

Schizophyllum commune and *Cerrena* sp. were among the most frequently isolated basidiomycete fungi from discoloured wood of living *Acacia* hybrid stems. *Schizophyllum commune* is often considered a sap-rot basidiomycete (Schmidt 2006; Takemoto et al. 2010) but is also described as a saprobe (Nicolotti et al. 1998; Padhiar et al. 2010), associated with sapwood rot or heart rot in more than 70 species of landscape trees including acacia, eucalypt, ash and tamarind (Hickman et al. 2011). This fungus is also recognised as a cause of heart rot (Visarathononth 1990; Oprea et al. 1994). *S. commune* was isolated from both sites in this study but was not detected by direct sequencing of DNA from *Acacia* wood at Phan Truong Hai. In vitro wood decay tests of lignin biodegradation showed that *S. commune* could cause

minor weight loss, which increased after one month (Koyani et al. 2016). A recent study by Trang (2017 unpublished) showed that *S. commune* was less aggressive than *Cerrena* sp., which produced lesions triple the length of lesions caused by *S. commune* 35 days after artificial inoculation in four *Acacia* hybrid clones. Fruiting bodies of *Cerrena* sp. and *Schizophylum commune* which were confirmed by rDNA sequencing were also found in dead branches of *Acacia* hybrid at the Nghia Trung site (July 2012). These are potential inoculum sources for wood decay at this site but most likely do not represent a significant threat to living trees.

Pseudolagarobasidium acaciicola was among the most frequently isolated basidiomycetes and was isolated from both sites but detected in only two of the pooled DNA samples from Phan Truong Hai.. This fungus has been reported to cause a root rot resulting in dieback of *A. cyclops*, an invasive weed in South Africa (Wood & Ginns 2006; Kotzé et al. 2015). Trang (2017 unpublished) showed that the length of the under bark lesion length produced by *P. acaciicola* was the same as that of *Cerrena* sp. but three-fold greater than *S. commune* isolates 35 days after artificial inoculation into four *Acacia* hybrid clones. This fungus may potentially be a root rot pathogen, though no disease has, to our knowledge, yet been attributed to *P. acaciicola* in Vietnam. *Acacia cyclops* is the only host species for *P. acaciicola* recorded in the USDA-ARS fungal databases (https://nt.ars-grin.gov/fungaldatabases/ accessed 24/3/2018).

The number of ascomycete OTUs identified by 454 method was 1.6-fold higher than that of basidiomycete fungus taxa, almost half of which were yeasts and not regarded

as significant in wood decay processes. This ratio reinforces the results from isolation by which ascomycete species were three-fold greater than basidiomycete species. As with the wood decay basidiomycetes, there was low overlap in fungal species detected by environmental sequencing and those detected by isolation; only 11 OTUs were detected by both methods.

This study found no significant difference between fungal communities associated with discoloured wood and those associated with decayed wood. It is possible that the fungi responsible for discolouration and decay are minor components of the mycota associated with living stems of *Acacia* hybrid trees and any differences are masked by more abundant endophytes when total fungal communities are analysed. This indicates that results from isolating onto a basidiomycete-selective medium may be more informative for investigating differences in fungi that are minor components of the mycota. Though isolations were made from both sites in this study, they were only from discoloured wood samples and not decayed wood samples as decayed wood is more likely to be infected with secondary wood-rotting fungi that are more combative than the primary colonisers (Hiscox et al. 2018).

Although there were no main fungi responsible for discolouration and/or decay, this study showed that several fungi were present which have the potential to damage *Acacia* hybrid stem quality. The geographic and host range of *Pseudolagarobasidium acaciicola* in Vietnam also warrants scrutiny to determine whether it represents a potential threat to *Acacia* plantations in Vietnam.

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Chapter 5

Screening for host responses in *Acacia* to a canker and wilt pathogen, *Ceratocystis manginecans*

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Summary

In Vietnam, the productivity of *Acacia* hybrid (*Acacia mangium* x *A. auriculiformis*) plantations is being threatened by an aggressive canker pathogen, *Ceratocystis manginecans* and selection for tolerance is the main control strategy. A pot trial was established in Binh Duong province to screen for the host response of nine *Acacia* genotypes (six *Acacia* hybrid clones, two *A. auriculiformis* clones and mixed provenance seedlings of *A. mangium*) to artificial inoculation with three isolates of *C. manginecans*. Lesion lengths as measured on the inner bark suggested that the two *A. auriculiformis* clones were relatively more tolerant to *C. manginecans* than the *A. mangium* genotype. In contrast, the lesion lengths of all six *Acacia* hybrid clones fell between the *A. auriculiformis* and *A. mangium* genotypes. The results of this study indicate that among the *Acacia* hybrid clones, BV10 showed the most tolerance to *C. manginecans*. Chemical analysis of crude sapwood extracts sampled from the lesion provided some evidence that induced phenolic compounds, particularly tetrahydroxyflavanone and condensed tannins may have a defensive role in the *Acacia – C. manginecans* pathosystem. However, results were not consistent across individual *Acacia* hybrid clones and *A. mangium* genotypes.

5.1 Introduction

Over the last decade, a vascular wilt and stem canker disease caused by a species of *Ceratocystis* has become the most damaging disease of *Acacia*, especially *A. mangium*, causing large scale mortalities in Indonesia, Vietnam and Malaysia (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015). First described in Indonesia as *C. acaciivora* Tarigan & M. van Wyk (Tarigan et al. 2011a), recent molecular studies have identified this pathogen as *C. manginecans* M. van Wyk, Al-Adawi & M.J. Wingf. (Fourie et al. 2015). Other authors consider that several recently described new species of *Ceratocystis* including *C. acaciivora* and *C. manginecans*, are populations within a large species complex for which the most appropriate name is *C. fimbriata* Ellis & Halst. (Oliveira et al. 2015). By 2015, this *Ceratocystis* wilt and canker pathogen was affecting approximately 2000 ha of *Acacia* plantations across Vietnam (Plant Protection Department - MARD 2015). A recent study estimated that the incidence of this disease on *A. auriculiformis*, *A. mangium* and *Acacia* hybrid plantations ranged from 7.1 – 12.5%, 9.2 – 18.4% and 10.2 – 18.2%, respectively (Thu et al. 2016).

Clones of *Acacia* hybrid, the natural hybrid between *Acacia auriculiformis* Benth. and *A. mangium* Willd, are the most widespread plantation species established in Vietnam (Beadle et al. 2013a) with a total of approximately 400,000 ha planted (Nambiar & Harwood 2014). Although *Acacia* hybrid is largely grown to supply the domestic demand for pulpwood and wood chips for the export market (Bueren 2004; Nambiar et al. 2015), a significant proportion of the *Acacia* hybrid estate is

increasingly being managed for solid wood, mainly for furniture (Kha et al. 2012). Silvicultural practices required to produce solid wood from *Acacia* include singling, pruning and thinning (Trang et al. 2017). Wounds thus created have been shown to facilitate the entry of pathogens including *C. manginecans* (Tarigan et al. 2011b).

Phenolic compounds such as stilbenes, flavonoids, lignans and tannins have been shown to play a major role in tree defence following fungal invasion in some woody plants (Pearce 1996; Eyles et al. 2003a; Tsai et al. 2006; Wallis et al. 2008). The phenolic chemistry of *A. auriculiformis* and *A. mangium* heartwood has been examined previously, however, these studies focused on heartwood extractives (Barry et al. 2005b; Mihara et al. 2005; Barry et al. 2006). In functional sapwood, infection is restricted by inducible defence mechanisms including de novo accumulation of polyphenols and changes in water content; in contrast, heartwood, comprising of dead cells, is protected by high levels of biochemicals that inhibit fungal colonisation (Pearce 1996).

This paper investigated the host responses of nine *Acacia* plantation genotypes to three isolates of the canker and wilt pathogen, *C. manginecans.* This study aimed to link host tolerance, as indicated by lesion size with the localised accumulation of phenolic chemistry (i.e. condensed tannins, total phenolics as well as eight selected individual phenolic compounds) in the sapwood of all *Acacia* genotypes. To our knowledge, this is the first paper to characterize the phenolic profile induced by fungal inoculation in the sapwood of *Acacia* species. Understanding potential

chemical markers of tolerance or susceptibility could be of value for determining *Acacia* hybrid clones showing higher host tolerance to fungal attack.

5.2 Materials and methods

5.2.1 Plant material

A total of nine *Acacia* genotypes comprising two *A. auriculiformis*, six *Acacia* hybrids and mixed provenance seedlings of *A. mangium* were used in this study. Full details of the genetic history of each *Acacia* genotype are detailed in Table 5-1.

Table 5-1: Genetic background of the selected *Acacia* planting material.

Taxon	Genotype number	Origin of genetic material	Note
Acacia auriculiformis	AA1	FORTIP trial in Binh Duong (Nghia & Chien 2007b).	AA1 and AA9: recognised as superior clones by the Ministry of Agricultural and Rural Development (MARD) of Vietnam in Decision No: 3377/QĐ-BNN-TCLN dated 16/12/2010.
	AA9	AA9 trial in Dong Nai (Nghia & Chien 2007b).	
Acacia hybrid (*A. mangium* x *A. auriculiformis*)	BV10	Mother = *A. mangium* Daintree (Queensland, Australia) provenance. Father = *A. auriculiformis* Darwin (Northern Territory, Australia) provenance (Kha 2000).	BV10: recognised as a superior clone by MARD in Decision No: 132/QĐ/BNN-KHCN dated 17/1/2000.
Acacia hybrid	BV33	Mother = *A. mangium* Daintree (Queensland, Australia) provenance.	BV33: recognised as a superior clone by MARD in Decision No: 1998/QĐ/BNN-KHCN dated 11/7/2006.

Taxon	Genotype number	Origin of genetic material	Note
		Father = *A. auriculiformis* Darwin (Northern Territory, Australia) provenance (Kha 2000).	
	AH1	*Acacia* hybrid plantations in Dong Nai and Binh Duong, Vietnam (Nghia & Chien 2007a).	AH1 and AH7: recognised as superior clones by MARD in Decision No: 3905/QĐ-BNN-TCLN dated 11/12/2007.
	AH7	*Acacia* hybrid plantations in Dong Nai and Binh Duong, Vietnam (Nghia & Chien 2007a).	
Acacia hybrid	TB12	Mother = Mossman (Queensland, Australia) provenance Father = possibly Oenpelli (Northern Territory, Australia) provenance (Chis Harwood pers.comm.).	TB6 and TB12: recognised as superior clones by MARD in Decision No: 3118/QĐ/BNN-KHCN dated 9/8/2000.
	TB6	Mother = Mossman (Queensland, Australia) provenance	

Taxon	Genotype number	Origin of genetic material	Note
		Father = possibly Oenpelli (Northern Territory) provenance (Chris Harwood pers.comm.).	
Acacia mangium (seedlings)	AM	Mixed provenance seedlings - Papua New Guinea (PNG).	Seeds were imported from PNG by Institute of Forest Tree Improvement and Biotechnology.

5.2.2 Fungal material

Three *C. manginecans* cultures isolated from *Acacia* hybrid trees in Vietnam were selected as inoculum (Table 5-2). The identities of *C. manginecans* were determined from DNA sequence data of the rDNA ITS and β-tubulin genes. DNA fragments were amplified using primers ITS1-F/ITS4 (White et al. 1990) and Bt1a/Bt1b (Glass & Donaldson 1995), respectively. All isolates are stored at the Vietnamese Academy of Forest Sciences. Cultures were prepared by subculturing from stock culture to PDA in 90-mm-diameter Petri dishes and incubating at room temperature (25 °C) for 15 days.

Table 5-2: Genbank accession numbers for ITS and β-tubulin sequences of *Ceratocystis manginecans* isolates.

Species	Isolate	ITS accession #	β-tubulin accession #
Ceratocystis mangenicans	C1	MF033455	MF040712
Ceratocystis mangenicans	C2	MF033456	MF040713
Ceratocystis mangenicans	C3	MF033457	MF040714

5.2.3 Pot trial site and experiment design

The pot trials were located at Bau Bang station, Binh Duong province, southern Vietnam (Latitude: 11º27′74.3″N and Longitude: 106º63′35.5″E). The climate in southern Vietnam is characterised by distinct dry and wet seasons, the latter receiving > 90% of the total annual rainfall of 1500 – 2500 mm from May to November; the mean annual temperature is 27.6 –28.6 °C with little monthly variation. In June 2013, 32 clonally replicated trees from each of eight *Acacia* clones provided by the South-eastern Forest Research and Experimental Centre and 32 seedling trees of *A. mangium* provided by the Institute of Forest Tree Improvement and Biotechnology were planted in 20 cm diameter pots. In September 2013, the trees were transferred to 50 cm diameter pots. Pots were spaced 1 x 1.5 m apart. Each pot was irrigated daily with 3 L of water using an automatic irrigation water system.

The experiment was set up as a randomised complete block design, with five treatments for each of the nine *Acacia* genotypes and four blocks (replicates). Fungal treatments consisted of three isolates of *C. mangenicans* (C1, C2 and C3) and two types of controls (mock wounded and unwounded trees) served as references, giving a total of 20 trees per *Acacia* genotype.

The diameters (at 1.3 m tree height above pot surface) and heights of trees were measured once, just prior to inoculation. All trees of each of the genotypes were of similar diameter (3.76 ± 0.11 cm; mean ± standard error) and height (491 ± 8 cm).

5.2.4 Experimental fungal inoculation

In August 2014, 14-month-old trees were inoculated with a fungal isolate on the stem 50 cm above the soil. In brief, the bark was removed with a sterile borer (10 mm diameter) and a 10 mm diameter PDA plug, either colonized with 15-day-old mycelia (fungal inoculation) or sterile [mock inoculation: to control for potential effects of wounding alone on induced responses (Eyles et al. 2007)] was placed mycelium-side down onto the cambium. The wounds were wrapped with Parafilm to retain the inoculum and limit desiccation and contamination.

5.2.5 Lesion length assessment

Host resistance was based on lesion length, which is an appropriate estimate of relative host resistance in this and other canker and heart rot systems (Blodgett et al. 2007; Guimaraes et al. 2010a; Brawner et al. 2015). Trees were destructively harvested 23 days after inoculation with three *C. manginecans* isolates. The lesion length that developed over bark (OB) was measured first and then the bark was removed to measure the under bark (UB) lesion length.

5.2.6 Wood extraction and analysis of phenolic compounds

An 80-cm length of stem centred on the inoculation site was cut from the main stem. This stem length was halved longitudinally through the inoculation wound with a blade (Figure 5-1). A cordless drill was used to obtain shavings of sapwood from the following locations:

- in inoculated treatment — the infected region (Figure 5-1),
- in wounded control treatment — 1 cm above the inoculation site,
- in unwounded control treatment — at a similar height to the other treatments.

Drill bits were sterilised with ethanol (70%) and flamed for 30 seconds between each sampling. Fresh shavings (0.5 mg) were extracted twice with 1 mL of 100% grade methanol over 24 hours in the dark at 4 °C. The pooled extracts were transferred to a 2 mL tube and stored in a freezer (-20 °C) until transported to the University of Tasmania under quarantine permit (IP14010539) and then stored at - 80 °C in a freezer until analysed.

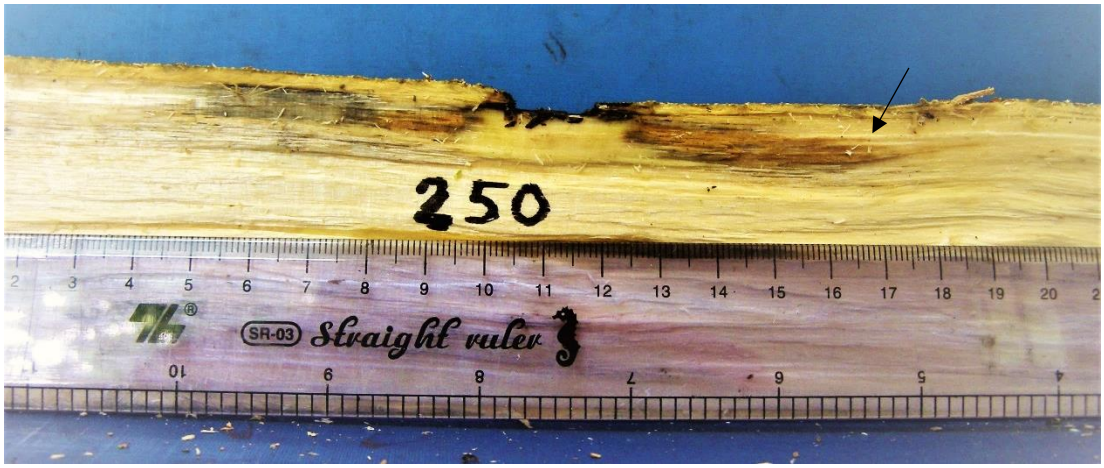

Figure 5-1: Representative photo showing inoculation wound and lesion caused by *Ceratocystis mangenicans* on *Acacia* hybrid (BV33) observed on sapwood. Black arrow indicates where the tissue was sampled.

Samples were analysed by UPLC-UV-MS using a Waters Acquity H-series UPLC coupled to a Waters Acquity Photo Diode Array (WAPDA) detector connected in series with a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (2.1 x 100 mm x 1.7 µ particles) was used. The solvents were 1% acetic acid in water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 0.35 mL/min, with initial conditions of 98%A: 2%B for 0.5 min then a linear ramp to 44%A:56%B at 15 minutes, followed by a linear ramp to 5 %A:95 %B at 20 min, with a 1 min hold at the final value before re-equilibration for 3 minutes to initial conditions. Injection volume was 2.5 µL. The WAPDA was monitored from 230 nm to 500 nm at a resolution of 1 nm and data for quantitative measurements were extracted at 280 nm. A small number of peaks observed on the 280 nm chromatogram were selected for individual quantitation. Condensed tannin response was estimated from the area of the 'hump' observed underneath all the individually eluting phenolic compounds by subtracting the area of all individual peaks from the area of the whole chromatogram.

The mass spectrometer was operated in negative ion electrospray ionisation mode with needle voltage of 2.8 KV, scanning from *m/z* 120 to 1200 every 0.25 s with a cone voltage of 45 V. Phenolic compounds potentially present based on previous studies on *Acacia* heartwood were also initially monitored by Selected Ion Monitoring (SIM) at *m/z* 271, 287, 289, 303, 305 and 479, with 35 ms dwell time on each ion. The ion source temperature was 130 °C, the desolvation gas was nitrogen at 950 L/hr, the cone gas flow was 50 L/hr and the desolvation temperature was 450 °C. Data were analysed using MassLynx and TargetLynx software.

Reference standards of teracacidin and 2,3-trans-3,4',7,8-tetrahydroxyflavanone were available. Eight individual phenolic compounds were measured with reference to a catechin (Merck) standard curve (1-20 µg mL^{-1} dissolved in acetone) and results were expressed as catechin equivalent per mg fresh weight of wood.

Individual phenolic compounds 1 to 8 were denoted as Cp1 to Cp8, respectively. They were observed at 4.20, 5.02, 5.68, 5.92, 6.52, 6.80, 7.40 and 8.33 minutes, respectively (Figure 5-2).

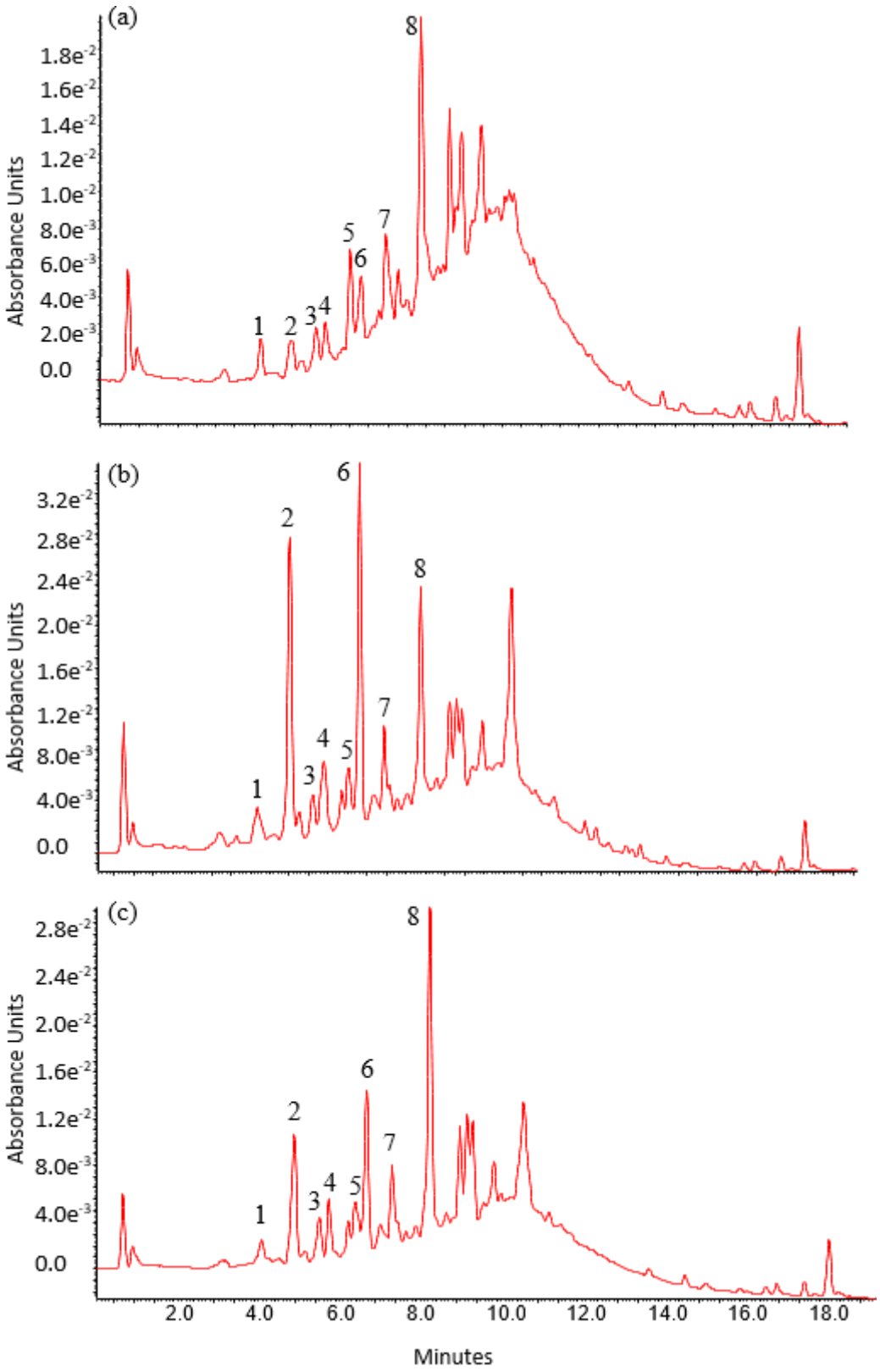
(a)
Absorbance Units
1.8e-2
1.6e-2
1.4e-2
1.2e-2
1.0e-2
8.0e-3
6.0e-3
4.0e-3
2.0e-3
0.0
1
2
3
4
5
6
7
8
(b)
Absorbance Units
3.2e-2
2.8e-2
2.4e-2
2.0e-2
1.6e-2
1.2e-2
8.0e-3
4.0e-3
0.0
1
2
3
4
5
6
7
8
(c)
Absorbance Units
2.8e-2
2.4e-2
2.0e-2
1.6e-2
1.2e-2
8.0e-3
4.0e-3
0.0
1
2
3
4
5
6
7
8
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
Minutes

Figure 5-2: HPLC-UV chromatogram (280 nm) of a 100% methanol extract of (a) *Acacia auriculiformis*, (b) *A. mangium* and (c) *Acacia* hybrid (TB12) 23 days after inoculation with *Ceratocystis manginecans* isolate C1. Identities of peaks are as follows: 1, unknown flavonoid; 2, 2,3 -trans 3,4',7,8 tetrahydroxyflavanone; 3, unknown flavonoid; 4, a tetrahydroxyflavanone; 5, unknown flavonoid; 6, putative 4',7,8 trihydroxyflavanone; 7, unknown flavonoid; 8, unknown flavonoid.

5.2.7 Statistical analysis

Two *Acacia* genotypes, BV10 and BV33, were characterised by very thick bark and exploratory analysis of the OB lesion lengths for these genotypes showed that they were very short compared to the UB lesion lengths (i.e. mean OB lesion lengths were 3.3 and 2.8 cm whereas averaged UB lesion lengths were 16.0 and 20.8 cm, respectively for BV10 and BV33). As such, UB rather OB lesion lengths were used to examine treatment effects – previous screening trials of *Ceratocystis* sp. have similarly measured lesions formed under the bark (Roux et al. 2004; Brawner et al. 2015).

Two-way analysis of variance (ANOVA) was used to test the effects of block, *Acacia* genotype, fungal isolates and the interactions of genotype and isolate on diameter, height, lesion length and phenolic chemistry (total phenolic concentration, condensed tannins and eight selected phenolic compounds).

The concentrations of phenolic compounds in both the mock wounded and unwounded control treatments were very low compared with the inoculated treatment, therefore the treatment effects were examined for inoculated trees only. Full details of effect of treatment on constitutive chemistry are presented in Appendices (Appendices 5-1 to 5-3). The assumptions of ANOVA such as homogeneity of variance and the Gaussian distribution were evaluated by the use of quantile – quantile plots and residual plots for all variables. Only the phenolic data required log transformation to produce normalised distributions of residuals. Fisher's protected least significant difference post hoc tests were used to determine significant differences among treatment means. All analyses were performed using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC, USA).

5.3 Results

5.3.1 Relative host response of nine *Acacia* genotypes to inoculation with three *C. manginecans* isolates

Lesion length was significantly influenced by *Acacia* species and genotype (Figure 5-3 and Table 5-3). The lesion lengths induced by fungal inoculation in *A. mangium* were significantly longer than those induced in *A. auriculiformis*, while the lesions induced in the six *Acacia* hybrid clones were intermediate in length. Of the six *Acacia* hybrid clones, the lesion length of BV10 was most similar to the *A. auriculiformis* genotype AA1.

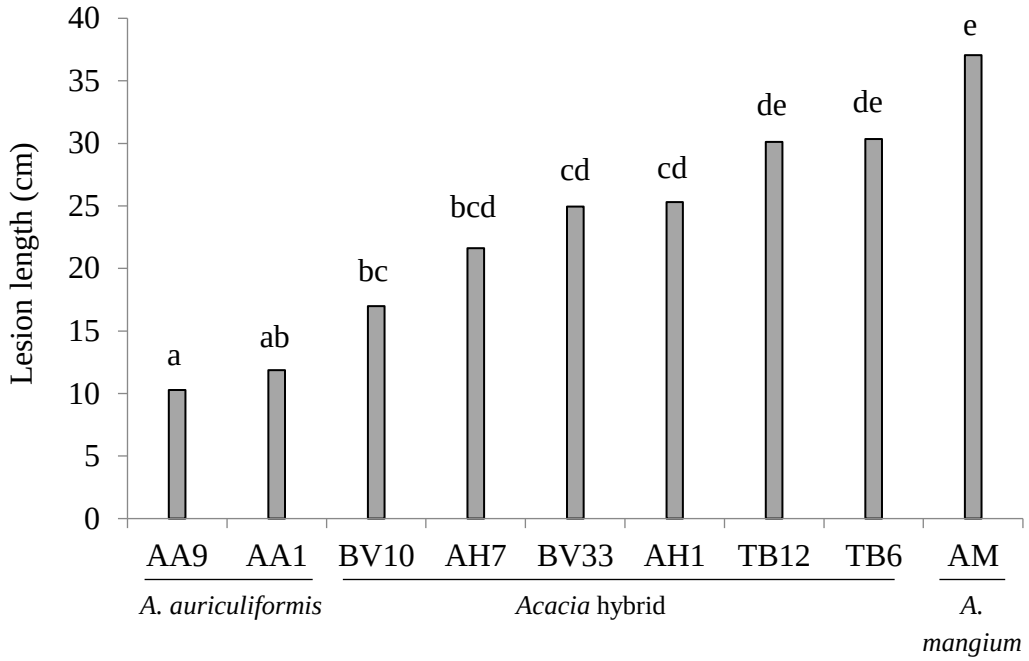

Figure 5-3: Effect of *Acacia* genotype on lesion lengths 23 days after inoculation with three *Ceratocystis manginecans* isolates. Different letters show significant differences at $p < .001$ (N = 12 trees). See Table 5-1 for details of *Acacia* genotypes.

Lesion length was significantly affected by fungal isolate (Table 5-3). Lesion length induced by isolate C3 was significantly longer by 93.1% and 36.6% than that of isolate C2 and C1, respectively. Lesion length of isolate C2 was significantly longer by 41.4% than that of isolate C1 (Figure 5-4).

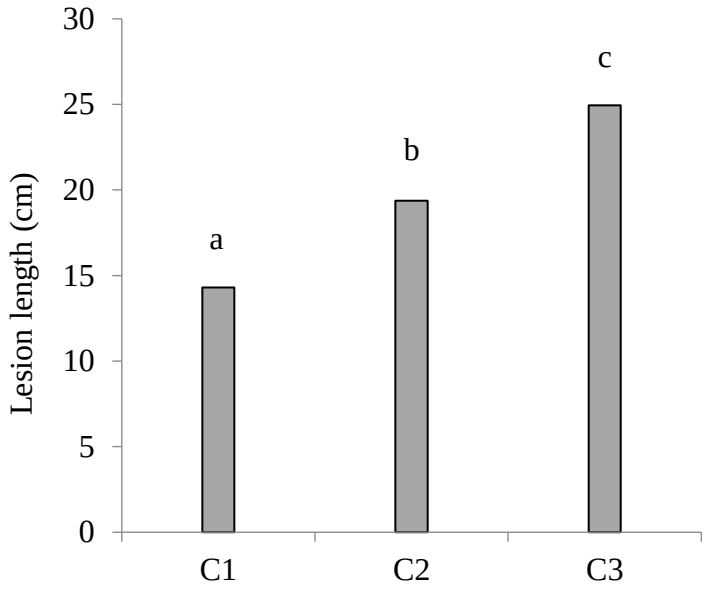

Figure 5-4: Effects of *Ceratocystis manginecans* isolates (C1, C2 and C3) on lesion lengths 23 days after inoculation on nine *Acacia* genotypes (results from clones combined). Different letters show significant differences at $p < .001$ (N = 36 trees).

Table 5-3: Summary of a two-way ANOVA that examined the effects of nine *Acacia* genotypes and three *Ceratocystis manginecans* isolates on lesion lengths and concentrations of induced phenolic compounds. The two controls were not included in this analysis, N = 4.

Response variables*	*Acacia* genotypes *p*-value	*C. manginecans* isolates *p*-value	*Acacia* genotype x fungal isolate *p*-value
Lesion length	<0.001	<0.001	0.20
Total peaks	0.01	0.06	0.10
Condensed tannins	0.003	0.20	0.08
Cp1	<0.001	0.07	0.08
Cp2	<0.001	0.52	0.02
Cp3	<0.001	0.06	0.06
Cp4	<0.001	0.01	0.17
Cp5	<0.001	0.40	0.03
Cp6	0.05	0.20	0.12
Cp7	0.28	0.84	0.05
Cp8	0.008	0.50	0.20

*See Table 5-4 for details of phenolic compounds Cp1 – 8.

5.3.2 Characterisation of phenolic compounds

Analysis of *Acacia* crude wood extracts by UPLC-UV-MS indicated the presence of a complex range of phenolic compounds (Figure 5-2 and Table 5-4). The identity of Cp2 was unequivocally confirmed as 2,3 –trans 3,4',7,8 tetrahydroxy flavanone by

direct comparison with a standard. Other related flavanones, Cp4 and Cp6 (putative 4',7,8 trihydroxyflavanone), were identified on the basis of UV, MS, and tandem MS evidence only and not by comparison with authentic standards. The other five phenolic compounds were tentatively identified as unknown flavonoids.

With the exceptions of Cp6 and Cp7, phenolic chemistry was significantly influenced by *Acacia* species and genotype (Table 5-3 and Table 5-5). The concentrations of total peaks, condensed tannins and seven compounds (except Cp2) were similar for *A. auriculiformis* and *A. mangium*. Among all of the *Acacia* genotypes, BV10 had the highest concentrations of total peaks, condensed tannins, and Cp1, Cp3, Cp4, Cp5 and Cp8.

Fungal isolates did not affect the concentrations of total peaks, condensed tannins and seven phenolic compounds with the exception of Cp4 (Table 5-3). Concentration of Cp4 (a tetrahydroxyflavanone) induced by *C. manginecans* isolate C3 was significantly lower than that induced by isolates C1 and C2 by approximately 65% and 53%, respectively (Figure 5-5).

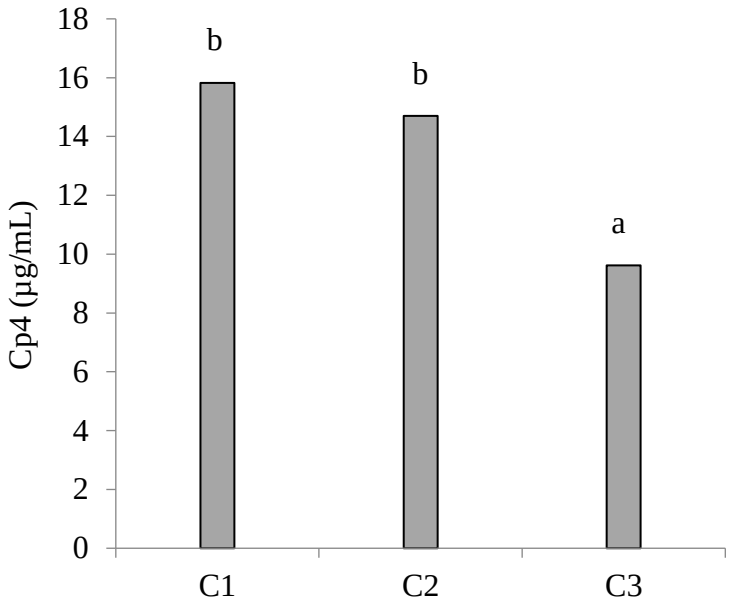

Figure 5-5: Effects of *Ceratocystis manginecans* isolates (C1, C2 and C3) on concentrations of phenolic compound Cp4 (a tetrahydroxyflavanone) extracted from the sapwood of *Acacia* trees (results combined from 9 clones). Different letters show significant differences at $p < .001$ (N = 36 trees).

Table 5-4: Characterisation of eight selected phenolic compounds from the crude wood extracts of *Acacia* genotypes after infection with *Ceratocystis manginecans*.

Phenolic compounds	Molecular weight	UV maxima	Tentative identification
Cp1	286	289	Unknown flavonoid
Cp2	288	294	2,3 -trans 3,4',7,8 tetrahydroxyflavanone*
Cp3	318	286	Unknown flavonoid
Cp4	288	289	a tetrahydroxyflavanone
Cp5	302	286	Unknown flavonoid
Cp6	272	293	Putative 4',7,8 trihydroxyflavanone
Cp7	286	284	Unknown flavonoid
Cp8	328	277	Unknown flavonoid

Table 5-5: Effects of nine *Acacia* genotypes on the induced phenolic chemistry 23 days after inoculation with *Ceratocystis manginecans*. Values shown are the means of concentrations (µg/mL) of 12 trees. Means with different letters in the same row are significantly different ($p < .05$).

Phenolic	*A. auriculiformis*		*Acacia* hybrid						*A. mangium*
compounds*	AA1	AA9	AH1	AH7	BV10	BV33	TB12	TB6	AM
Total peaks	251.3^{a}	237.8^{a}	265.1^{a}	294.8^{ab}	435.5^{c}	407.3^{bc}	249.0^{a}	318.1^{abc}	277.6^{a}
Condensed tannins	502.5^{de}	472.1^{cde}	371.4^{a}	451.9^{abcde}	543.5^{e}	429.5^{abcd}	380.1^{ab}	381.8^{abc}	462.4^{bcde}
Cp1	7.4^{ab}	6.4^{ab}	7.9^{ab}	8.1^{ab}	37.3^{d}	22.4^{cd}	6.8^{ab}	12.1^{bc}	6.1^{a}
Cp2	13.3^{ab}	10.1^{a}	34.0^{d}	25.8^{bcd}	15.1^{abc}	73.8^{e}	30.4^{cd}	32.6^{d}	33.7^{d}
Cp3	6.3^{bc}	6.3^{bc}	3.1^{a}	6.4^{bc}	24.6^{e}	13.4^{d}	4.8^{ab}	9.1^{cd}	4.0^{ab}
Cp4	12.1^{bcd}	12.9^{cd}	6.2^{a}	12.8^{bcd}	62.8^{e}	20.7^{d}	6.7^{ab}	13.3^{cd}	7.0^{abc}

Phenolic compounds*	*A. auriculiformis*		*Acacia* hybrid						*A. mangium*
	AA1	AA9	AH1	AH7	BV10	BV33	TB12	TB6	AM
Cp5	8.0^{c}	8.2^{c}	3.7^{a}	6.7^{bc}	19.1^{d}	9.0^{c}	4.4^{ab}	6.8^{bc}	5.5^{abc}
Cp6	18.4	18.8	21.3	27.4	29.8	43.6	21.5	31.2	33.9
Cp7	11.5	11.4	9.8	12.0	9.1	15.0	10.1	11.1	15.6
Cp8	24.8^{ab}	32.7^{ab}	39.7^{bc}	43.5^{bc}	53.7^{c}	28.7^{a}	34.7^{ab}	35.6^{ab}	33.7^{ab}

*See Table 5-1 and Table 5-4 for details of *Acacia* genotypes and phenolic compounds Cp1 – 8, respectively

5.4 Discussion

In this study, lesion lengths in response to inoculation with *C. manginecans* varied significantly among *Acacia* genotypes. These data indicated that *A. auriculiformis* was significantly more tolerant to *C. manginecans* than *A. mangium*, and this response was consistent for all three isolates of *C. manginecans*. Since the discovery of *C. manginecans*, there has been a series of resistance screening trials with *Acacia* in Indonesia, Malaysia and Vietnam (Tarigan et al. 2011a; Thu et al. 2012; Chen et al. 2013; Brawner et al. 2015; Tarigan et al. 2016). Levels of tolerance to *C. manginecans* in *A. mangium* are low and resistance is rarely observed but other species such as *A. auriculiformis* show greater tolerance. The lesion length of the five *Acacia* hybrid clones in this study fell between the two *A. auriculiformis* clones and *A. mangium* genotypes, confirming that a gradient of tolerance exists in hybrids.

Reports of host tolerance or resistance for the same *Acacia* genotype have not always been consistent. For example, in our study of young trees, *C. manginecans* elicited lesions in AH1 and AH7 but these same genotypes appeared resistant in a previous field trial (Nghia et al. 2013). This inconsistency may indicate ontogenetic variation in resistance or tolerance as indicated in artificial inoculation trials at a young age may not be indicative of field tolerance at a later age when trees are exposed to predisposing conditions that may promote disease such as regular wounding by animals, high loads of inoculum and strains with different virulence. In our study, lesion length indicated that isolate C3 was the most aggressive while isolate C1 was the least aggressive of the three isolates, regardless of *Acacia* genotype. A wide

variation in the pathogenicity of *C. manginecans* has been shown in other studies such as Thu et al. (2012).

Given the observed higher degree of tolerance of *A. auriculiformis* and its hybrids to *C. manginecans* and the variation in the response to three isolates, we hypothesised that these differences could be related to the induction of phenolic compounds, as has been previously reported in many woody tree species (Barry et al. 2005b; Mihara et al. 2005; Woodward et al. 2007; Sherwood & Bonello 2013; Chen et al. 2014; Araujo et al. 2016). The inverse relationship between lesion length and Cp4 concentration provides some evidence, although correlational, that the induction of this compound may have a defensive role in the *Acacia – C. manginecans* pathosystem. However, although significant differences in phenolic profiles were generally demonstrated among the *Acacia* genotypes regardless of *C. manginecans* isolate, the changes in the concentrations of the eight selected phenolic compounds and total phenolic compounds did not consistently relate well to the observed variation in host tolerance, indicated by lesion size variation. Although the lesion lengths of the *Acacia* hybrid clones ranged between the *A. auriculiformis* clones and *A. mangium* genotypes, the concentrations of phenolic compounds of the *Acacia* hybrid clones were, in general, similar or higher than those observed for *A. auriculiformis* clones and *A. mangium* genotypes. For example, the concentrations of total peaks, Cp1, Cp3, Cp4, Cp5 and Cp8 in BV10 were significantly higher than in either the *A. auriculiformis* clones or *A. mangium* genotypes.

Phenolic Cp2, identified as 2,3-trans 3,4',7,8 tetrahydroxyflavanone, has been previously recorded at significantly higher levels in the heartwood of *A. auriculiformis* compared to *A. mangium* (Barry et al. 2005b; Mihara et al. 2005; Barry et al. 2006). This compound showed antifungal activity against *Phellinus noxius* and *P. badius* using *in vitro* bioassays (Mihara et al. 2005) and it was suggested that it accounted for the lower susceptibility of *A. auriculiformis* to heart rot. However, Cp2 did not appear to be associated with tolerance to *C. manginecans* as concentrations induced in sapwood were higher in *A. mangium* compared to *A. auriculiformis*. Cp2 was even detected in sapwood of *A. mangium* (0.16 µg/mL) in unwounded control trees (Appendix 5-3). A pathogen isolate which is virulent on a given host can overcome, in some way, the chemical defences of that host. However given the observed higher degree of tolerance of *A. auriculiformis* and its hybrids to *C. manginecans* and the variation in the response to three isolates, we hypothesised that these differences could be related to the induction of phenolic compounds, as has been previously reported in many woody tree species (Barry et al. 2005b; Mihara et al. 2005; Barry et al. 2006).

There was a trend for higher concentrations of condensed tannins associated with shorter lesions, e.g. the concentrations of condensed tannins in AA1, AA9 and BV10 clones were significantly higher than in TB6 and TB12 whereas an opposite pattern was observed for the lesion length. The accumulation of condensed tannins as an indicator of tolerance to *Ceratocystis* pathogens has been previously described (El Modafar et al. 1996; Brignolas et al. 1998; Hammerbacher et al. 2014) and for *A. auriculiformis* and *Acacia* hybrid BV10, condensed tannins may have a defensive

role in the *Acacia* – *C. manginecans* pathosystem. The high concentrations of condensed tannins in *A. mangium* may appear to contradict the involvement of condensed tannins in defence but the effects of these tannins will depend on their presise nuture. Those accumulated in *A. mangium* may be of a different type to the condensed tannins in the more tolerant *Acacia* genotypes.

This pioneer study has revealed some promising phenolic markers for investigating host responses in *Acacia* to invasion by fungi although more research is required to understand the phenolic chemistry associated with host tolerance. We can confirm that a clear gradient of tolerance to *C. manginecans*, as indicated by lesion lengths, exists in *Acacia* species. This variation must be fully exploited, especially the transference of tolerance from *A. auriculiformis* to *A. mangium* through hybridisation. *Acacia* hybrid, the natural hybrid between *A. mangium* and *A. auriculiformis*, is a key multipurpose plantation species that is increasingly being planted across Vietnam for both sawn timber and pulpwood products.

Acknowledgments

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Chapter 6

Screening for host responses in *Acacia* to three white rot basidiomycete fungi

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Initially, this experiment was writen as a part of the paper "Screening for host responses in *Acacia* to a canker and wilt pathogen, *Ceratocystis manginecans,* and to three basidomycetous fungi" submitted to Forest Pathology Journal. However, it was suggested by reviewers that this work should be described separately and it is included here as a chapter.

Summary

A proportion of *Acacia* hybrid plantations in Vietnam are managed for timber product. However, they appear susceptible to fungal associated stem defects and the host response to commonly occurring fungi was investigated. Artificial inoculation of three basidiomycete species (*Pseudolagarobasidium acaciicola*, *Cerrena* sp. and *Schizophyllum commune*) were undertaken with four *Acacia* genotypes. Lesion lengths developed following inoculation with white rot species were similar across the four *Acacia* genotypes examined, regardless of species. However, lesion lengths of *P. acaciicola* and *Cerrena* sp. were significantly longer than those of *S. commune*. For basidiomycete species, host tolerance to artificial inoculation as assessed by lesion length appeared unrelated to the induction of phenolic compounds.

6.1 Introduction

The secondary plant surface (periderm and rhytidome) provides a barrier preventing the entry of most potential pathogens, and constitutive and induced defence mechanisms in the bark (cortex and phloem) protect the wood (xylem) from microbial attack (Pearce 1996). A few pathogens are able to penetrate these outer tissues directly, but most xylem pathogens gain entry through wounds that expose this tissue and make it more vulnerable to attack. There are six broad groups of xylem pathogens: wood decaying fungi, canker rots, endophytes and latent infections, vascular wilts, xylem-inhabiting bacteria, and viruses (Pearce 1996). This experiment investigated the responses of *Acacia* to wounding and artificial inoculation with three white rot basidiomycete fungi.

Clones of *Acacia* hybrid, the natural hybrid between *Acacia auriculiformis* Benth. and *A. mangium* Willd, are the most widespread plantation species established in Vietnam (Beadle et al. 2013) with a total of approximately 400,000 ha planted (Nambiar & Harwood 2014). Although *Acacia* hybrid is largely grown to supply the domestic demand for pulpwood and wood chips for the export market (Bueren 2004; Nambiar et al. 2015), a significant proportion of the *Acacia* hybrid estate is increasingly being managed for solid wood, mainly for furniture (Kha et al. 2012). Silvicultural practices required to produce solid wood from *Acacia* include singling, pruning and thinning. Wounds thus created may facilitate the introduction of decay fungi, leading to stem defects and discolouration (Trang et al. 2017) and negatively impact the quality of timber.

High levels of heart rot have been observed in *Acacia mangium* (Mahmud et al. 1993; Ito & Nanis 1997; Barry et al. 2002b; Bougher & Tommerup 2002; Mihara et al. 2005) whereas *A. auriculiformis* is considered less prone to heart rot (Ito & Nanis 1997; Ito 2002; Barry et al. 2005b; Mihara et al. 2005). A recent field study showed that pruning and thinning increased the incidence and severity of discoloration and decay in a three-year old *Acacia* hybrid plantation in Vietnam (Trang et al. 2017) indicating that *Acacia* hybrid may have similar susceptibility to wood decay fungi as its parent *A. mangium*.

In host-pathogen interactions, phenolic compounds have been shown to play a major role in chemical defence following fungal invasion in some woody plants (Pearce 1996; Eyles et al. 2003a; Tsai et al. 2006; Wallis et al. 2008). A range of compounds including hydrolysable tannins, polymeric proanthocyanidins, flavonoid glycosides and formylated phloroglucinol compounds were detected in the bark lesion margin of three-year-old *Eucalyptus globulus* Labill. in response to a natural fungal infection by *Cytonaema* sp. (Eyles et al. 2003b). In temperate plantations of pruned *E. nitens* (Deane & Maiden) Maiden, the reaction zone typically contained four- to six-fold more polyphenolic compounds than the sound sapwood (Barry et al. 2000; Barry et al. 2001), although the amount was influenced by the extent of wood decay caused by the different decay fungi present (Barry et al. 2002a). Higher levels of condensed tannins and teracacidin were found in the heartwood of *A. auriculiformis* compared with *A. mangium* (Barry et al. 2005b; Mihara et al. 2005).

This experiment investigated the host responses of nine *Acacia* plantation genotypes to three white rot basidiomycete species (*Pseudolagarobasidium acaciicola* Ginns, *Cerrena* sp. and *Schizophyllum commune* Fr.). A recent study found these three basidiomycete species were amongst the most frequently isolated basidiomycetes from the discoloured and rotted wood of *Acacia* hybrid plantations at Nghia Trung and Phan Truong Hai in South Vietnam (Trang et al., unpublished data). This study aimed to link host tolerance, as indicated by lesion size with the localised accumulation of phenolic chemistry (i.e. condensed tannins, total phenolics as well eight selected individual phenolic compounds) in the sapwood of all *Acacia* genotypes. Understanding potential chemical markers of tolerance or susceptibility could be of value for determining *Acacia* hybrid clones showing higher host tolerance to fungal attack.

6.2 Materials and methods

6.2.1 Plant material

Initially a total of nine *Acacia* genotypes comprising two *A. auriculiformis*, six *Acacia* hybrids and mixed provenance seedlings of *A. mangium* were used in this study. However, only four *Acacia* genotypes including AA1, AA9, BV10 and BV33 were used in this study as other genotypes had been destroyed by squirrels. Full details of the genetic history of each *Acacia* genotype are provided in Table 5-1.

6.2.2 Fungal material

Three basidiomycete fungal species isolated from *Acacia* hybrid trees in Vietnam were selected as inoculum (Table 6-1). The identities of the three white rot basidiomycete species were determined from DNA sequence data of the rDNA ITS. DNA fragments were amplified using primers ITS1-F/ITS4 (White et al. 1990). All isolates are stored at the Vietnamese Academy of Forest Sciences. Cultures were prepared by subculturing from stock culture to PDA in 90-mm-diameter Petri dishes and incubating at room temperature (25 °C) for 15 days.

Table 6-1: Genbank accession numbers for ITS sequences of the three white rot basidiomycete species.

Species	ITS accession number
Pseudolagarobsidium acaciicola	MF033455
Schizophyllum commune	MF033456
Cerrena sp.	MF033457

6.2.3 Pot trial site and experiment design

The pot trials were located at Bau Bang station, Binh Duong province, southern Vietnam (Latitude: 11º27′74.3″N and Longitude: 106º63′35.5″E). The climate in southern Vietnam is characterised by distinct dry and wet seasons, the latter receiving > 90% of the total annual rainfall of 1500 – 2500 mm from May to

November; the mean annual temperature is 27.6 –28.6 °C with little monthly variation. In June 2013, 32 clonally replicated trees from each of eight *Acacia* clones provided by the South-eastern Forest Research and Experimental Centre and 32 seedling trees of *A. mangium* provided by the Institute of Forest Tree Improvement and Biotechnology were planted in 20 cm diameter pots. In September 2013, the trees were transferred to 50 cm diameter pots. Pots were spaced 1 x 1.5 m apart. Each pot was irrigated daily with 3 L of water using an automatic irrigation water system.

The experiment was set up as a randomised complete block design, with five fungal treatments for each of the nine *Acacia* genotypes and four blocks (replicates). Fungal treatments consisted of three white rot basidiomycete species (*P. acaciicola*, *Cerrena* sp. and *S. commune*) and two types of controls (mock wounded and unwounded trees), giving a total of 20 trees per *Acacia* genotype. However, a squirrel attack in May 2014 destroyed five (AH1, AH7, TB12, TB6 and AM) of the nine *Acacia* genotypes and therefore it was not possible to include them in the trial. Therefore, this experiment assessed four *Acacia* clones (AA1, AA9, BV10 and BV33).

The diameters (at 1.3 m tree height above pot surface) and heights of trees were measured once, just prior to inoculation. All trees of each of the genotypes were of similar diameter (3.80 ± 0.18 =cm; mean ± standard error) and height (500 ± 10 cm).

6.2.4 Experimental fungal inoculation

In August 2014, 14-month-old trees were inoculated with a fungal isolate on the stem 50 cm above the soil. In brief, the bark was removed with a sterile borer (10 mm diameter) and a 10 mm diameter PDA plug colonized with 15-day-old mycelia (fungal inoculation) or no fungi (mock inoculation: to control for potential effects of wounding alone on induced responses (Eyles et al. 2007) was placed mycelium-side down onto the cambium. The wounds were wrapped with Parafilm to retain the inoculum and limit desiccation and contamination.

6.2.5 Lesion length assessment

Host resistance was based on lesion length, which is an appropriate estimate of relative host resistance in this and other canker and heart rot systems (Blodgett et al. 2007; Guimaraes et al. 2010a; Brawner et al. 2015). Trees were destructively harvested 35 days after inoculation with three white rot basidiomycete species. The lesion length that developed over bark (OB) was measured first and then the bark was removed to measure the under bark (UB) lesion length.

6.2.6 Wood extraction and analysis of phenolic compounds

Wood extraction and analysis of phenolic compounds were done as in “Wood extraction and analysis of phenolic compounds” section in Chapter 5.

6.2.7 Statistical analysis

Statistical analysis methods were done as in "Statistical analysis" section in Chapter 5.

6.3 Results

6.3.1 Relative host response of nine *Acacia* genotypes to inoculation with three white rot basidiomycete fungi

Lesion length was not affected by *Acacia* clones but was significantly influenced by fungal species. In particular, the lesion length of *S. commune* was three-fold shorter than either *P. acaciicola* or *Cerrena* sp. (Figure 6-1).

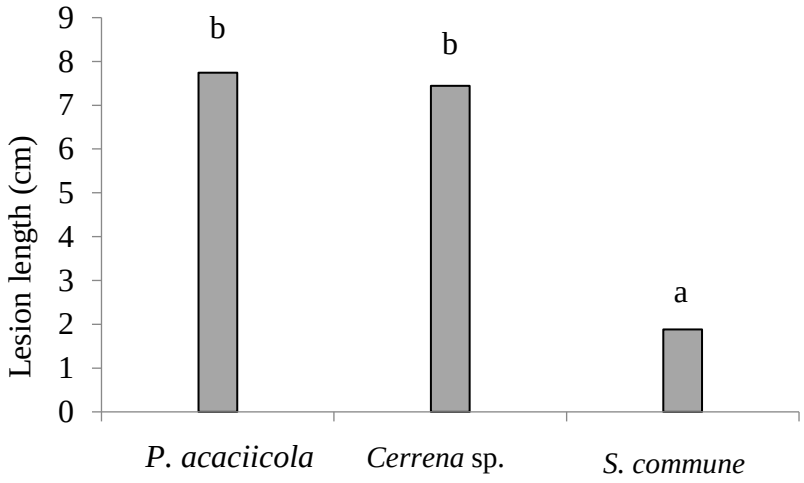

Figure 6-1: Effect of three basidiomycete fungal isolates on lesion lengths 35 days after inoculation on four *Acacia* hybrid clones. Different letters show significant differences at $p < .001$ (N = 12 trees).

6.3.2 Characterisation of phenolic compounds

Phenolic compounds in this experiment were the same as those in the "Screening for host responses in *Acacia* to a canker and wilt pathogent, *Ceratocystis manginecans*", Chapter 5.

The concentrations of Cp2, Cp4 and Cp8 were significantly affected by *Acacia* hybrid genotype (Figure 6-2). In particular, the concentrations of Cp4 and Cp8 in BV10 were similar to AA1 but significantly higher than AA9 and BV33 (Figure 6-2 b and c). BV33 had significantly higher concentration of Cp2 than AA1, AA9 and BV10 (Figure 6-2 a).

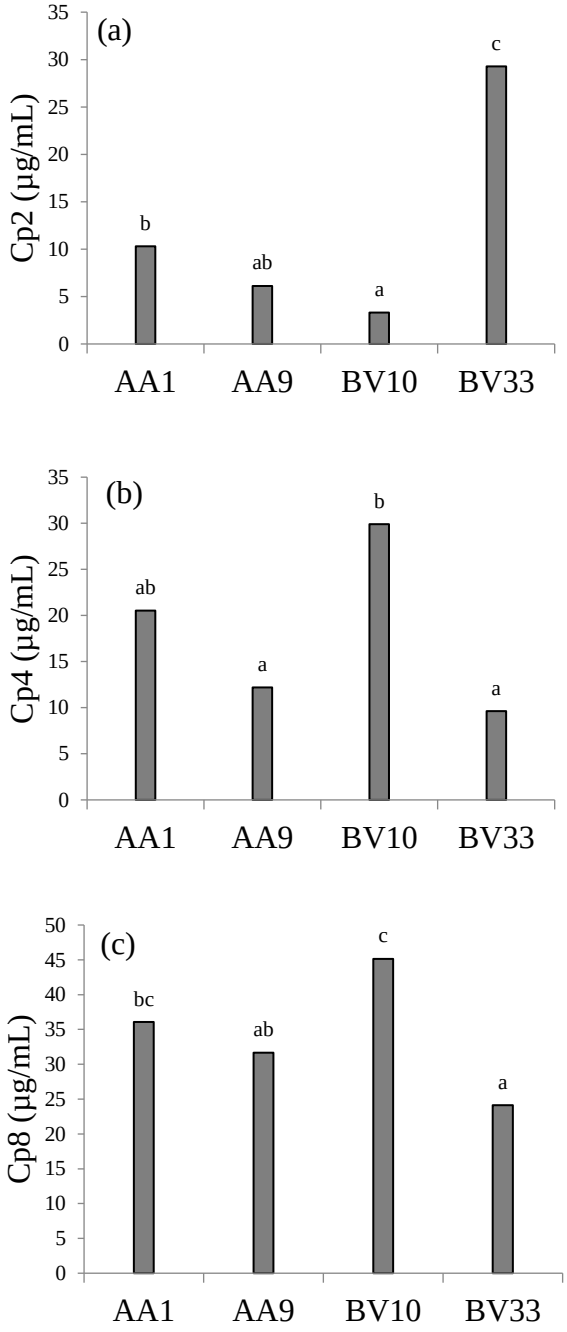

Figure 6-2: The concentrations of phenolic compounds Cp2 (a), Cp4 (b) and Cp8 (c) extracted from the sapwood of four *Acacia* genotypes, 35 days after inoculation with three basidiomycete wood-rotting species. Different letters show significant differences at $p < .01$ (a and c) and $p < .05$ (b), N = 9 trees.

The phenolic chemistry was significantly affected by fungal species and *Acacia* genotype but not affected by their interaction (Table 6-2). There were no significant differences in the concentrations of total peaks, condensed tannins and eight induced phenolic compounds between *P. acaciicola* and *Cerrena* sp. (Table 6-2). However, the concentrations of total peaks, condensed tannins and seven induced phenolic compounds after inoculation with those two species were higher than produced by inoculation with *S. commune*. Specifically, concentrations of condensed tannins and total peaks were approximately 2 – 3 fold higher after inoculation with *P. acaciicola* and *Cerrena* sp. than with *S. commune*. Similarly, concentrations of phenolic compounds were 3 – 10 fold higher after inoculation with *P. acaciicola* and *Cerrena* sp. than with *S. commune*.

Table 6-2: Summary of a two-way ANOVA that examined the effects of four *Acacia* genotypes (AA1, AA9, BV10 and BV33) and three white rot basidiomycete species on lesion length and concentrations of induced phenolic compounds. The two controls were not included in this analysis, N = 3.

Response variables*	*Acacia* genotype *p*-value	Basidiomycete species *p*-value	*Acacia* genotype x fungal species *p*-value
Lesion length	0.2	<0.001	0.7
Total peaks	0.5	<0.001	0.6
Condensed tannins	0.1	<0.001	0.8
Cp1	0.2	<0.001	0.6
Cp2	0.002	<0.001	0.5
Cp3	0.3	<0.001	0.7
Cp4	0.028	<0.001	0.5
Cp5	0.2	0.002	0.8
Cp6	0.1	<0.001	0.2
Cp7	0.6	0.003	0.7
Cp8	0.005	ns	0.5

*See Table 5-4 for details of phenolic compounds Cp1 – 8.

Table 6-3: Effects of three white rot basidiomycete species on induced phenolic chemistry 35 days after inoculation. Values are means of concentrations (µg/mL) from 12 trees. Means with the same letter in the same row are not significantly different ($p < .05$).

Phenolic Compounds*	Basidiomycete species		
	P. acaciicola	*Cerrena* sp.	*S. commune*
Total peaks	506.0[b]	565.2[b]	173.9[a]
Condensed tannins	670.4[b]	726.8[b]	376.6[a]
Cp1	44.3[b]	42.2[b]	5.4[a]
Cp2	13.4[b]	21.1[b]	2.2[a]
Cp3	22.4[b]	24.7[b]	2.6[a]
Cp4	23.9[b]	28.6[b]	6.2[a]
Cp5	16.9[b]	14.1[b]	3.9[a]
Cp6	13.9[b]	19.1[b]	1.8[a]
Cp7	7.6[b]	13.1[b]	2.6[a]
Cp8	29.6	34.8	36.1

*See Table 5-4 for details of phenolic compounds Cp1 – 8.

6.4 Discusion

The three basidiomycete species in this study were frequently isolated from stem discolouration associated with mechanical wounding (pruning and/or thinning) in *Acacia* hybrid plantations in South Vietnam (unpublished data). To our knowledge, there is no prior record of artificial inoculation of living tree species with these basidiomycete species. There was no variation in host response to the three basidiomycete rot isolates, i.e. across all 4 genotypes *P. acaciicola* and *Cerrena* sp.

elicited longer and similar lesion lengths compared with *S. commune.* Although the number of genotypes that could be screened was small (two clones of *A. auriculiformis* and two *Acacia* hybrid clones) due to squirrel damage and did not include *A. mangium*, the concentrations of phenolic compounds were consistently much lower after inoculation with *S. commune* than with *P. acaciicola* or *Cerrena* sp., indicating the host response in the *Acacia* genotypes was related to the pathogenicity of the fungal isolate.

Lesion development induced by these basidiomycetes was relatively limited when compared to that induced by the highly pathogenic vascular wilt pathogen *C. manginecans* (Figure 5-3 and Figure 6-1). Of the three basidiomycete species in our study, only *P. acaciicola* is reported as a tree pathogen, causing dieback disease in *A. cyclops*, an invasive weed in South Africa (Wood & Ginns 2006; Kotzé et al. 2015). In other tree species *P. acaciicola* has been reported as an endophyte. The *Cerrena* species was unknown but the closely related *Cerrena unicolor* has been reported to be associated with stem decay in species of *Tilia*, *Betula* and *Acer* in Finland (Terho et al. 2007; Terho & Hallaksela 2008). *Schizophyllum commune* is most often described as a saprobe (Nicolotti et al. 1998; Padhiar et al. 2010), associated with sapwood rot or heart rot in more than 70 species of landscape trees including acacia, eucalypt, ash and tamarind (Hickman et al. 2011).

Pearce (1996) stated that most wood decay fungi colonise only functionally compromised sapwood (major wounds) and are opportunists, and that they may function as weak pathogens (e.g. *P. acaciicola* and *Cerrena* sp.) or have little or no

ability to invade living host tissues (e.g. *S. commune*). As suggested by Barry et al. (2006) our studies confirm that tests of sapwood resistance do not infer what might occur in heartwood, e.g. *S. commune* is well known as a heart-rot agent (Visarathononth 1990; Oprea et al. 1994). The management of these opportunistic and weakly pathogenic decay fungi resides in the avoidance of major wounds and infection courts (Trang et al. 2017) and the exploration of host tolerance is of more promise with primary fungal rot pathogens (e.g.(Woodward et al. 2007; Baietto & Wilson 2010; Nemesio-Gorriz et al. 2016).

Chapter 7

General discussion

This chapter overviews and summarises the major findings of the study in the context of its significance to management of *Acacia* hybrid plantations in Vietnam. Recommendations for further research are given as well as recommendations for management.

7.1 Silvicultural practices affect wood quality of *Acacia* hybrid plantations

Acacia hybrid (*Acacia mangium* × *A. auriculiformis*) is widely planted in Vietnam and part of the estate is managed for solid timber products (Kha et al. 2012; Nambiar et al. 2015). This requires silvicultural practices including pruning and thinning (Medhurst et al. 2002; Pinkard et al. 2004) that through mechanical wounding can facilitate the entry of fungal organisms, leading to stem defects (Han & Kellogg 2000; Barry 2002). A destructive survey was conducted in a 3-year-old *Acacia* hybrid plantation at Nghia Trung in Binh Phuoc province, 18 months after the imposition of pruning and thinning treatments. The results of this study showed that pruning increased the incidence and severity of discoloration and decay in stems of *Acacia* hybrid trees even though pruning was carried out in the dry season which is considered less conducive to fungal infection (Lee & Arentz 1997). This may be due to the pruning of live branches with basal diameter greater than 2 cm as this has been

found to increase decay incidence and severity in temperate eucalypts (Pinkard et al. 2004). Therefore, in order to reduce the impact of pruning on *Acacia* wood quality the pruning of live branches with basal diameter greater than 2 cm must be avoided as much as practicable. Tip pruning rather than complete removal of these larger branches is recommended.

Thinning also increased the incidence of stem decay and the severity of discoloration but not the severity of decay. The development of discoloration and decay may be due to the wounds caused during the thinning activity which is commonly associated with thinning in forestry (Hunt & Krueger 1962; White & Kile 1991). In addition, strong wind events resulting in branch breakages occurred at Nghia Trung in the period between the application of treatments and sampling, with a higher prevalence of broken branches in the thinned stands compared to unthinned (Pham Van Bon; pers. comm.). However, more study is required to confirm the origin of wounds associated with thinning. In this study, trees were sampled only once, at the age of 18 months, therefore this sampling period may not have been sufficiently long to assess the full effect of thinning on the severity of decay. For example, in an *E. nitens* plantation, decay column length assessed five years after pruning was 2-3 fold longer than one year after pruning (Barry et al. 2005a).

In another experiment, the *Acacia* hybrid trees at Phan Truong Hai that were harvested at year 3 also showed some levels of discolouration and decay (unpublished data) and there was a potential for further development of discolouration up to harvest age at 7 years old. However, the preliminary results of

discolouration and decay of *Acacia* hybrid at Phan Truong Hai showed that there was no increase in discolouration and decay between years three and seven (unpublished data) it is suggested that in these *Acacia* hybrid trees at Phan Truong Hai, healthy wood grew rapidly and that the fungi were not effective rotters as found in Chapter 4, therefore the proportions of discolouration and decay compared to log surface area did not appear to increase.

7.2 Fungal agents associated with stem defects and potential basidiomycete and ascomycete fungi causing stem defects

The presence of fungal decay agents in *Acacia* hybrid trees was examined in the two Vietnamese plantations. In July 2011, just prior to a second thinning, discoloured wood samples were taken from a three-year old *Acacia* hybrid plantation at Phan Truong Hai for the isolation of fungi. And in July 2012, approximately 18 months after pruning and thinning treatments, discoloured wood samples were taken from a three-year old *Acacia* hybrid plantation at Nghia Trung for the isolation of fungi. The 231 isolates were classified into 22 different members of Ascomycota and 13 Basidiomycota. *Ceratocystis manginecans* was isolated from both sites. *Ceratocystis manginecans* has been reported to cause severe disease in *Acacia* species, especially *A. mangium* in Indonesia, Vietnam and Malaysia (Tarigan et al. 2011a; Thu et al. 2012; Brawner et al. 2015). Three of the *C. manginecans* isolates from this study were pathogenic when tested against nine *Acacia* genotypes and were more aggressive than three basidiomycete isolates. Some identified basidiomycete isolates have the potential to cause heart rot, e.g. *Phellinus noxius* (Lin et al. 2013; Burcham

et al. 2015) and *Pseudolagarobasidium acaciicola* (Wood & Ginns 2006; Kotzé et al. 2015), or sap-rot, e.g. *Schizophyllum commune* (Schmidt 2006; Takemoto et al. 2010). Recently, Thu (2016a) showed that the ambrosia beetle, *Euwallacea fornicatus*, a new record and potential insect pest, occurred in stems of *A. auriculiformis*, *A. mangium* and *Acaci*a hybrid plantations over three years of age in Vietnam. This beetle carries a fungal symbiont identified as *Fusarium euwallaceae* which can infect and kill the tree. *Fusarium euwallaceae* was not found in this study, which examined trees up to three years of age.

In May 2015, approximately four years after thinning and fertilizer treatments, discoloured and decayed wood samples were taken from the above (seven-year old) *Acacia* hybrid plantation at Phan Truong Hai for fungal identification. DNA was extracted directly from discoloured and decayed wood samples and fungal rDNA ITS amplicons sequenced on a Roche 454 sequencer. The results showed that fungal community composition associated with discoloured and decayed wood of *Acacia* hybrid was not significantly affected by the thinning and fertilizer treatments or their interaction at the Phan Truong Hai plantation. A total of 109 fungal species were identified, including 68 members of Ascomycota and 41 Basidiomycota. The 41 Basidiomycota included 24 wood decay basidiomycetes. Combined with the 12 wood decay species isolated from the same sites, a total of 36 wood decay basidiomycetes were detected but only one of them was detected by both methods. The low overlap between fungi detected by isolation and those detected by environmental sequencing has been observed previously in wood (Jang et al. 2016). The most abundant fungi detected by direct sequencing in this study were members

of the Xylariaceae and are most likely endophytes that do not harm their hosts. The wood decay basidiomycetes were a minor component of the fungal communities in living *Acacia* hybrid trees in these two Vietnamese plantations. They accounted for under 2% of the total 454 sequence reads. This is consistent with the theory that wood decay basidiomycetes are held in stasis in living wood by osmotic or chemical conditions (Chapela & Boddy 1988). Taken together with the lack of increase in decay between three and seven years after pruning and thinning, these results indicate that the *Acacia* hybrid clones at these sites have innate defenses against heartrot fungi, including aggressive species such as *Phellinus noxius* and *Pseudolagarobasidium acaciicola.* Testing of new *Acacia* hybrid clones for tolerance or resistance against these heartrot agents as well as the canker and wilt pathogens *Ceratocystis manginecans* and *Fusarium euwallaceae* is therefore recommended before extensive deployment.

7.3 Chemicals associated with a defensive role in the *Acacia* - *Ceratocystis manginecans* and screening for *Acacia* hybrid clones tolerant to *C. manginecans* strategy

In Vietnam, the productivity of *Acacia* hybrid (*Acacia mangium* x *A. auriculiformis*) plantations is being threatened by an aggressive canker pathogen, *Ceratocystis manginecans* and selection for tolerance is the main control strategy (Gadgil et al. 2000). A pot trial was established in Binh Duong province to screen for the host response of nine *Acacia* genotypes (six *Acacia* hybrid clones, two *A. auriculiformis* clones and mixed provenance seedlings of *A. mangium*) to artificial inoculation with

three isolates of *C. manginecans* which were isolated from *Acacia* hybrid plantations at Nghia Trung and Phan Truong Hai. Measurements of lesion length demonstrated higher tolerance to *C. manginecans* in *A. auriculiformis* clones than in the *A. mangium* seedlings. The lesion lengths of all six *Acacia* hybrid clones fell between those of *A. auriculiformis* and *A. mangium* and BV10 was the most tolerant to *C. manginecans* among the six *Acacia* hybrid clones. These results are consistent with field observations (Nguyen Minh Chi, pers. comm.) and indicate that artificial inoculation of young, potted trees can be used for early selection of *Acacia* hybrid clones tolerant to *C. manginecans*. This artificial inoculation method has also been applied to selection of tropical *Acacia* species tolerant to *Ganoderma philippii* (Gafur et al. 2015).

Phenolic compounds have been shown to inhibit fungal invasion in woody plants (Pearce 1996; Zeneli et al. 2006). In this study, chemical analysis of crude sapwood extracts sampled from the lesion provided some evidence that induced phenolic compounds, particularly tetrahydroxyflavanone and condensed tannins may have a defensive role in the *Acacia – C. manginecans* pathosystem. However, results were not consistent across individual *Acacia* hybrid clones and *A. mangium* genotypes.

Three white rot basidiomycete fungi (*Pseudolagarobasidium acaciicola*, *Cerrena* sp. and *Schizophyllum commune* isolated from *Acacia* hybrid plantations at Nghia Trung and Phan Truong Hai) were artificially inoculated on four *Acacia* genotypes. Lesion lengths that developed following inoculation with white rot species were similar across the four *Acacia* genotypes examined, regardless of species. However, lesion

lengths of *P. acaciicola* and *Cerrena* sp. were significantly longer than those of *S. commune*. The artificial inoculation of the three white rot basidiomycete species to the host showed that induction of phenolic compounds in the hosts appeared unrelated to lesion lengths.

The inconsistent results and lack of correlation between chemical response and lesion length indicates that implementation of chemical screening to identify clones tolerant to either *Ceratocystis* or heart-rot is not feasible at this stage. Measurement of internal lesion length following inoculation of potted plants appears to be a suitable method that avoids the biosecurity risk associated with inoculation of larger trees in the field, though further validation by field trials, without artificial inoculation, is still required. Artificial inoculation is usually limited to one or a few isolates and different pathogen genotypes may differ in their clone specificities (Gomez-Zeledon et al. 2013) so that an exact correlation between artificial inoculations and field trials may be unattainable.

7.4 Recommendation for application of research results and future research in Vietnam

Our research examined heart rot incidence and risk in *Acacia* hybrid plantations at two sites in the south of Vietnam. The risk of heart rot impacting profitability of *Acacia* plantations grown for solid wood products at other sites with different environmental conditions across Vietnam still needs investigation.

In this research, we used a rapid, early screening method to select *Acacia* hybrid clones tolerant to *Ceratocystis manginecans*. This research demonstrated that *A. auriculiformis* is more tolerant to *C. manginecans* than *A. mangium* and among *Acacia* hybrid clones, BV10 was the most tolerant to *C. manginecans*. As resistance tends to break down after a few years (Nambiar & Harwood 2014), an ongoing screening program for *Acacia* hybrid clones tolerant to *Fusarium euwallaceae* as well as to *Ceratocystis manginecans* is needed to ensure the ongoing productivity and profitability of Vietnamese plantations.

We used next generation sequencing to investigate the fungal communities in a thinned *Acacia* hybrid plantation. As pruning is likely to provide more entry points for decay fungi, this technique should be used to investigate the fungal communities in pruned plantations.

Pruning wounds appear to be the infection court for fungi that cause discolouration in *Acacia* hybrid, therefore in future research, management of pruning wounds needs further investigation, in particular the size of branches that can be safely pruned. Thinning significantly increased the incidence of discolouration and decay below 1.5 m height but not above 1.5 m, therefore more investigation into the origin of wounds associated with thinning should be carried out.

More investigations on quantification of stem discolouration and decay should be carried out on older *Acacia* hybrid trees in order to identify the best time to harvest trees for sawlog purposes. As discussed in Chapter 3 a consistent rating system for

quantifying the impacts of management practices on the development of discolouration and decay is required and acceptable limitation of decay levels established to facilitate monitoring of *Acacia* hybrid wood for sawlogs.

Although *C. manginecans* was isolated several times from both sites the impact on *Acacia* hybrid plantations at Nghia Trung and Phan Truong Hai was negligible, despite reports of this fungus causing serious problems on *Acacia* plantations in SE Asia including Vietnam (Thu et al. 2012; Fourie et al. 2016). In order to maintain this level of tolerance, breeding programs need ongoing screening work to ensure that the selected *Acacia* hybrid clones are tolerant to *C. manginecans*. Induced phenolic compounds, particularly tetrahydroxyflavanone and condensed tannins may have a defensive role in the *Acacia – C. manginecans* pathosystem, but they were not consistent across individual *Acacia* hybrid clones and *A. mangium* genotypes. Future investigations could be conducted to confirm this result or a switch to molecular techniques may provide the necessary tools to screen *Acacia* clones at the genetic level. Marker development to ensure the transference of tolerance from *A. auriculiformis* to *A. mangium* through hybridisation would be useful for early selection in breeding programs. Transcriptome sequencing of tolerant and susceptible clones following artificial inoculation to identify genes associated with host resistance would be the first step towards this aim.

The successful management of heart rot and of the devastating pathogen *Ceratocystis manginecans* in *Acacia* plantations for solid wood production will require a transdisciplinary approach to successful management. Experts in practical

silvicultural methods, tree physiology, tree genetics, host defence and fungal pathogen biology must co-operate to unravel the interactions that result in the expression of disease.

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APPENDIX

Appendix 2-1: Causal agents of heart rot

Fungal species	Host species	Region	Reference
Amyloporia sinuosa (Fr.) Rajchenb., Gorjón & Pildain (Syn. *Antrodia sinuosa* (Fr.) P. Karst.)	Lemon trees	Arizona	(Bigelow et al. 1998)
Antrodia heteromorpha (Fr.) Donk.	*Picea sitchensis*	USA (Alaska)	(Hennon 1995)
Armillaria gallica Marxm. & Romagn.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Armillaria mellea (Vahl) P. Kumm.	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Armillaria mellea (Vahl) P. Kumm.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Armillaria sp.	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Armillaria sp.	Norway spruce	Swabian Alb	(Schonhar 1994)
Armillaria spp.	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Aurantiporus alborubescens (Bourdot & Galzin) H. Jahn	Danish beech	Denmark	(Heilmann-Clausen & Christensen 2004)

Fungal species	Host species	Region	Reference
Bjerkandera adusta (Willd.) P. Karst.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Bjerkandera adusta (Willd.) P. Karst.	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Buglossoporus quercinus (Schrad.) Kotl. & Pouzar (Syn. *Piptoporus quercinus* (Schrad.) P. Karst.)	*Quercus*		(Wald et al. 2004)
Camarops tubulina (Alb. & Schwein.) Shear	*Fagus* spp.	Denmark	(Heilmann-Clausen & Christensen 2004)
Cerrena unicolor (Bull.) Murrill	*Tilia* spp., *Betula* spp., *Acer* spp.	Finland	(Terho et al. 2007; Terho & Hallaksela 2008)
Chondrostereum purpureum (Pers.) Pouzar	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Cinereomyces lindbladii (Berk.) Jŭlich	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Coniophora eremophila Lindsey & Gilb.	Lemon trees	Arizona	(Bigelow et al. 1998)
Coniophora sp.	*Picea abies*	Finland	(Lannenpaa et al. 2008)
Coriolopsis gallica (Fr.) Ryvarden	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Daedalea quercina (L.) Pers.	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Daedalea quercina (L.) Pers.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Daedaleopsis confragosa (Bolton) J. Schröt.	*Quercus robur*	Croatia	(Zupanic et al. 2009)

Fungal species	Host species	Region	Reference
Daedaleopsis confragosa (Bolton) J. Schröt.	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Desarmillaria tabescens (Scop.) R.A. Koch & Aime (Syn. *Armillaria tabescens* (Scop.) Emel)	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Diplomitoporous rimosus (Murrill) Gilb. & Ryvarden	*Juniperus occidentalis ssp. occidentalis*	USA	(Knapp & Soule 1999)
Echinodontium tinctorium (Ellis & Everh.) Ellis & Everh.	*Abies* spp.	Canada, USA (north-western states)	(Lewis & Lindgren 1999); (Vasaitis 2013) and references therein
Echinodontium tinctorium (Ellis & Everh.) Ellis & Everh.	Conifers	USA (Oregon and Washington)	(Parks & Flanagan 2001)
Fistulina hepatica (Schaeff.) With.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Fomes fomentarius (L.) Fr.	*Alnus* spp., *Betula* spp., *Fagus* spp.	Temperate and boreal Northern Hemisphere	(Arhipova et al. 2012); (Vasaitis 2013) and references therein
Fomes fomentarius (L.) Fr.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Fomitiporia robusta (P. Karst.) Fiasson & Niemalä	*Quercus* spp.	Temperate Eurasia	(Vasaitis 2013) and references therein

Fungal species	Host species	Region	Reference
Fomitiporia robusta (P. Karst.) Fiasson & Niemelä (Syn. *Phellinus robustus* (P. Karst.) Bourdot & Galzin)	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Fomitiporia robusta (P. Karst.) Fiasson & Niemelä (Syn. *Phellinus texanus* (Murrill) A. Ames)	*Juniperus occidentalis ssp. occidentalis*	USA	(Knapp & Soule 1999)
Fomitopsis officinalis (Vill.) Bondartsev & Singer	Conifers	Temperate and boreal Asia and western North America	(Parks & Flanagan 2001); (Vasaitis 2013) and references therein
Fomitopsis pinicola (Sw.) P. Karst.	Conifers	Temperate and boreal Northern Hemisphere	(Hennon 1995); (Vasaitis 2013) and references therein
Fomitopsis pinicola (Sw.) P. Karst.	*Picea abies*	Finland	(Lannenpaa et al. 2008)
Fuscoporia ferruginosa (Schrad.) Murrill (Syn. *Phellinus ferruginosus* (Schrad.) Pat.)	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Ganoderma applanatum (Pers.) Pat.	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Ganoderma applanatum (Pers.) Pat.	*Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Ganoderma applanatum (Pers.) Pat.	*Acacia dealbata*	Australia	(Bakshi 1976)
Ganoderma applanatum (Pers.) Pat.	*Acacia auriculiformis*	Australia	(Old et al. 1996)

Fungal species	Host species	Region	Reference
Ganoderma applanatum (Pers.) Pat.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Ganoderma lucidum (Curtis) P. Karst.	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Ganoderma lucidum (Curtis) P. Karst.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Ganoderma orbiforme (Fr.) Ryvarden	*Elaeis guineensis*	Indonesia, Malaysia, Papua New Guinea	(Pilotti 2005); (Mohammed et al. 2014)
Ganoderma resinaceum Boud.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Ganoderma spp.	*Acacia* spp.	Malaysia	(Lee 1996)
Ganoderma spp.	*Acacia* spp.	Vietnam	(Nghia 2006, 2010, 2015; Thu 2016b)
Grifola frondosa (Dicks.) Gray	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Gymnopilus penetrans (Fr.) Murrill	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Hericeum sp.	*Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Hericium erinaceus (Bull.) Pers.	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Heterobasidion annosum (Fr.) Bref.	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)

Fungal species	Host species	Region	Reference
Hymenochaete rubiginosa (Dicks.) Lév.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Inonotus leporinus (Fr.) Gilb. & Ryvarden (Syn. *Onnia leporine* (Fr.) H. Jahn)	*Picea abies*	Finland	(Lannenpaa et al. 2008)
Inonotus obliquus (Fr.) Pilát	*Betula* spp.	Temperate and boreal Northern Hemisphere	(Vasaitis 2013) and references therein
Inonotus pachyphloeus (Pat.) T. Wagner & M. Fisch. (Syn. *Phellinus pachyphloeus* (Pat.) Pat.)	*Acacia* spp.	Vietnam	(Nghia 2006, 2010, 2015; Thu 2016b)
Ischnoderma resinosum (Schrad.) P. Karst.	*Fagus* spp.	Denmark	(Heilmann-Clausen & Christensen 2004)
Kretzschmaria deusta (Hoffm.) P.M.D. Martin	*Acer* spp., *Ausculus* spp., *Citrus* spp., *Fagus* spp., *Hevea brasiliensis*, *Tilia* spp., *Ulmus* spp.	Temperate, tropical and subtropical regions	(Guglielmo et al. 2012) and references therein
Laetiporus sulphureus (Bull.) Murrill	*Castanea sativa, Fraxinus excelsior, Larix decidua, Larix sibirica, Picea abies, Prunus avium, Quercus robur, Salix alba*	Germany, Russia, Switzerland	(Rogers et al. 1999)

Fungal species	Host species	Region	Reference
Laetiporus sulphureus (Bull.) Murrill	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Laetiporus sulphureus (Bull.) Murrill.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Laetiporus sulphureus (Bull.) Murrill (Syn. *Polyporus sulphureus* (Bull.) Fr.)	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	*Acacia mangium*	Brazil	(Halfeld-Vieira et al. 2006)
Lentinellus vulpinus (Sowerby) Kühner & Maire	*Fagus* spp.	Denmark	(Heilmann-Clausen & Christensen 2004)
Lentinus kaufmanii A. H. Smith	*Picea sitchensis*	USA (Alaska)	(Hennon 1995)
Lenzites betulina (L.) Fr.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Meripilus giganteus (Pers.) P. Karst.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Merulius sp.	*Picea sitchensis*	USA (Alaska)	(Hennon 1995)
Mycena galericulata (Scop.) Gray	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Niveoporofomes spraguei (Berk. & M.A. Curtis) B.K. Cui, M.L. Han & Y.C. Dai (Syn. *Polyporus spraguei* Berk. & M.A. Curtis)	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Oxyporus cf. *latemarginatus*	*Acacia mangium*	Malaysia	(Lee & Noraini Sikin 1999)

Fungal species	Host species	Region	Reference
Peniophora cinerea (Pers.) Cooke	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Phaeolus schweinitzii (Fr.) Pat.	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Phellinus badius (Cooke) G. Cunn. (Syn. *Fomes badius* Cooke)	*Acacia catechu*	India	(Bakshi 1957)
Phellinus chrysoloma (Fr.) Donk	*Picea* spp.	Temperate and boreal Europe	(Lannenpaa et al. 2008); (Vasaitis 2013) and references therein
Phellinus hartigii (Allesch. & Schnabl) Pat.	*Abies* spp., *Tsuga* spp.	Temperate Eurasia, boreal North America	(Hennon 1995); (Vasaitis 2013) and references therein
Phellinus igniarius (L.) Quél. (Syn. *Fomes igniarius* (L.) Fr.)	*Acer saccharum, Betula alleghaniensis, Fagus grandifolia, Populus tremuloides*	North America	(Ohman & Kessler 1964)
Phellinus noxius (Corner) G. Cunn.	*Acacia mangium*	Malaysia	(Lee 1996; Lee & Noraini Sikin 1999)
Phellinus noxius (Corner) G. Cunn.	*Myristica fatua, Dysoxylum samoense, Hibiscus tiliaceous*	Pacific Islands	(Brooks 2002)

Fungal species	Host species	Region	Reference
Phellinus tremulae (Bondartsev) Bondartsev & P.N. Borisov	*Populus tremuloides*	Western USA	(Witt 2010)
Phellopilus nigrolimitatus (Romell) Niemelä, T. Wagner & M. Fisch.	*Picea sitchensis*	USA (Alaska)	(Hennon 1995)
Phlebia radiata Fr.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Pholiota adiposa (Batsch) P. Kumm.	*Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Pholiota alnicola (Fr.) Singer (Syn. *Flammula alnicola* (Fr.) P. Kumm.)	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Pholiota aurivella (Batsch) P. Kumm.	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Pleurotus cornucopiae (Paulet) Rolland	*Fagus* spp.	Denmark	(Heilmann-Clausen & Christensen 2004)
Pleurotus ostreatus (Jacq.) P. Kumm.	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Polyporus sp.	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Poria hypobrunnea Petch (Syn. *Rigidoporus hypobrunneus* (Petch) Corner)	*Acacia mangium*	Malaysia	(Lee 1996; Lee & Noraini Sikin 1999)

Fungal species	Host species	Region	Reference
Porodaedalea pini (Brot.) Murrill (*Phellinus pini* (Brot.) Pilát)	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Porodaedalea pini (Brot.) Murrill (Syn. *Phellinus pini* (Brot.) Pilát)	*Pinus sylvestris*	Estonia	(Lohmus 2016)
Porodaedalea pini (Brot.) Murrill (Syn. *Phellinus pini* (Brot.) Pilát)	Conifers	USA (Oregon and Washington)	(Parks & Flanagan 2001)
Postia subcaesia (A. David) Jülich (Syn. *Tyromyces subcaesius* A. David)	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Pseudoinonotus dryadeus (Pers.) T. Wagner & M. Fisch. (Syn. *Inonotus dryadeus* (Pers.) Murrill)	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Pyrofomes demidoffii (Lév.) Kotl. & Pouzar	*Juniperus occidentalis ssp. occidentalis*	USA	(Knapp & Soule 1999)
Resinicium bicolor (Alb. & Schwein.) Parmasto	Norway spruce	Germany	(Schonhar 1996)
Spongipellis delectans (Peck) Murrill	Danish beech	Denmark	(Heilmann-Clausen & Christensen 2004)
Steccherinum ochraceum (Pers.) Gray	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Stereum gausapatum (Fr.) Fr.	*Quercus robur*	Croatia	(Zupanic et al. 2009)

Fungal species	Host species	Region	Reference
Stereum hirsutum (Willd.) Pers.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Stereum rameale (Schwein.) Burt	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Stereum sanguinolentum (Alb. & Schwein.) Fr.	*Picea abies*	Finland	(Lannenpaa et al. 2008)
Stereum sanguinolentum (Alb. & Schwein.) Fr.	*Abies* spp., *Picea* spp.	Canada	(Lewis & Lindgren 1999)
Stereum sanguinolentum (Alb. & Schwein.) Fr.	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon 1995)
Stereum subtomentosum Pouzar	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Subantrodia juniperina (Murrill) Audet (Syn. *Antrodia juniperina* (Murrill) Niemalä & Ryvarden)	*Juniperus occidentalis ssp. occidentalis*	USA	(Knapp & Soule 1999)
Tinctoporellus epimiltinus (Berk. & Broome) Ryvarden	*Acacia mangium*	Malaysia	(Lee & Noraini Sikin 1999)
Trametes coccinea (Fr.) Hai J. Li & S.H. He (Syn. *Pycnoporus coccineus* (Fr.) Bondartsev & Singer)	*Acacia* spp.	Vietnam	(Nghia 2006, 2010, 2015; Thu 2016b)
Trametes gibbosa (Pers.) Fr.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Trametes spp.	*Acacia* spp.	Vietnam	(Nghia 2006, 2010, 2015; Thu 2016b)
Trametes trogii Berk.	*Quercus robur*	Croatia	(Zupanic et al. 2009)
Trametes versicolor (L.) Lloyd	*Quercus robur*	Croatia	(Zupanic et al. 2009)

Fungal species	Host species	Region	Reference
Trametes versicolor (L.) Lloyd (Syn. *Polyporus versicolor* (L.) Fr.)	*Liquidambar styraciflua* and/or *Liriodendron tulipifera*	USA	(Shortle & Cowling 1978)
Trichaptum abietinum (Dicks.) Ryvarden	*Picea sitchensis, Tsuga heterophylla*	USA (Alaska)	(Hennon & DeMars 1997)
Volvariella bombycine (Schaeff.) Singer	*Fagus* spp.	Denmark	(Heilmann-Clausen & Christensen 2004)
Xanthoporia radiata (Sowerby) Ţura, Zmitr., Wasser, Raats & Nevo (Syn. *Inonotus radiatus* (Sowerby) Karst.)	*Alnus glutinosa*	Latvia	(Arhipova et al. 2012)
Xanthoporia radiata (Sowerby) Ţura, Zmitr., Wasser, Raats & Nevo (Syn. *Mensularia radiata* (Sowerby) Lázaro Ibiza)	*Alnus* spp.	Temperate Eurasia	(Vasaitis 2013) and references therein

Appendix 4-1: Fungal taxa associated with *Acacia* hybrid plantations in Vietam.

No.	Class	Order	Family	Genus	Species	Isolates obtained at		Genbank accession#
						NT	PTH	
	Ascomycota							
1	Dothideomycetes	Unclassified Botryosphaeriales		undet.	Botryosphaeriales sp.		1	MF860401
2	Eurotiomycetes	Eurotiales	Trichocomaceae	Talaromyces	*Talaromyces* sp.	1		MF860402
3	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	*Aspergillus terreus*	1		MF860403
4	Sordariomycetes	Glomerellales	Glomerellaceae	Colletotrichum	*Colletotrichum* sp.	1	1	MF860404
5	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	*Clonostachys* sp. 1	8	1	MF860405
6	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	*Clonostachys* sp. 2	1		MF860406
7	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	*Clonostachys* sp. 3	1		MF860407
8	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma* sp. 1	2		MF860408
9	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma* sp. 2	1		MF860409
10	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium* sp. 1	1		MF860410
11	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium* sp. 2	1		MF860411
12	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium* sp. 3	4	3	MF860412
13	Sordariomycetes	Hypocreales	Nectriaceae	undet.	Nectriaceae sp. 1	2		MF860413
14	Sordariomycetes	Hypocreales	Nectriaceae	undet.	Nectriaceae sp. 2	40		MF860414

No.	Class	Order	Family	Genus	Species	Isolates obtained at		Genbank accession#
						NT	PTH	
15	Sordariomycetes	Hypocreales	Nectriaceae	undet.	Nectriaceae sp. 3	1		MF860415
16	Sordariomycetes	Microascales	Ceratocystidaceae	Ceratocystis	*Ceratocystis manginecans*	4	2	*
17	Sordariomycetes	Xylariales	Sporocadaceae	Neopestalotiopsis	*Neopestalotiopsis* sp.	1	10	MF860416
18	Sordariomycetes	Xylariales	Sporocadaceae	Pestalotiopsis	*Pestalotiopsis* sp. 1		13	MF860417
19	Sordariomycetes	Xylariales	Sporocadaceae	Pestalotiopsis	*Pestalotiopsis* sp. 2	11	4	MF860418
20	Sordariomycetes	Xylariales	Sporocadaceae	Pseudopestalotiopsis	*Pseudopestalotiopsis* sp.	29	19	MF860419
21	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	Debaryomyces	*Debaryomyces* sp. cf. *fabryi*		1	MF860420
22	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	Meyerozyma	*Meyerozyma*/*Candida* sp.	1		MF860421
	Basidiomycota							
23	Agaricomycetes	Agaricales	Psathyrellaceae	undet.	*Psathyrellaceae* sp. 1		2	MF621966
24	Agaricomycetes	Agaricales	Schizophyllaceae	Schizophyllum	*Schizophyllum commune*	3	6	KY930913
25	Agaricomycetes	Polyporales	undet.	undet.	Polyporales sp.	5	1	MF621967
26	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Phellinus	*Phellinus noxius*	1		MF621969
27	Agaricomycetes	Polyporales	Phanerochaetaceae	Phlebiopsis	*Phlebiopsis flavidoalba*	1		MF621970
28	Agaricomycetes	Polyporales	Phanerochaetaceae	Phlebiopsis	*Phlebiopsis* sp.	1		MF621971
29	Agaricomycetes	Polyporales	Phanerochaetaceae	Pseudolagarobasidium	*Pseudolagarobasidium acaciicola*	2	2	KY990563

No.	Class	Order	Family	Genus	Species	Isolates obtained at		Genbank accession#
						NT	PTH	
30	Agaricomycetes	Polyporales	Polyporaceae	Cerrena	*Cerrena* sp.	12		MF033437
31	Agaricomycetes	Polyporales	Polyporaceae	undet.	Polyporaceae sp.1	3		MF621972
32	Agaricomycetes	Polyporales	Polyporaceae	Trametes	*Trametes* aff. *cubensis*	1		MF621973
33	Agaricomycetes	Polyporales	Polyporaceae	undet.	Polyporaceae sp.2	9	1	MF621974
34	Agaricomycetes	Russulales	Peniophoraceae	Peniophora	*Peniophora* cf. *lycii*	4		MF621975
	Mucoromycota							
35		Mucorales	Cunninghamellaceae	Gongronella	*Gongronella* sp. cf. *butleri*	6		MF860422

Abbreviations: NT= Nghia Trung; PTH = Phan Truong Hai; *: ITS accession number: MF033455 to MF033457; β-tubulin accession number: MF040712 to MF040714;

Appendix 4-2: The direct identification of fungi associated with *Acacia* hybrid plantation at Phan Truong Hai by Roche 454 sequencer

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
	Ascomycota								
1	Dothideomycetes	Capnodiales	Capnodiaceae	Capnodium	*Capnodium* sp.	0.20	1	0	MF942470
2	Dothideomycetes	Capnodiales	Capnodiaceae	Leptoxyphium	*Leptoxyphium* sp.	1.82	3	2	MF942471
3	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 1	0.15	4	5	MF942472
4	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 2	0.19	1	2	MF942473
5	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 3	0.35	1	3	MF942474
6	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 4	0.14	2	6	MF942475
7	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 5	0.16	2	3	MF942476
8	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 6	10.54	8	9	MF942477
9	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium* sp. 7	0.79	2	2	MF942478
10	Dothideomycetes	Capnodiales	Cladosporiaceae	Toxicocladosporium	*Toxicocladosporium* sp.	0.35	1	3	MF942479
11	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Pallidocercospora	*Pallidocercospora* sp.	0.14	0	3	MF942480
12	Dothideomycetes	Capnodiales	Neodevriesiaceae	Neodevriesia	*Neodevriesia* sp.	0.21	0	1	MF942481
13	Dothideomycetes	Capnodiales	Teratosphaeriaceae	Parateratosphaeria	*Parateratosphaeria* sp.	0.22	2	2	MF942482
14	Dothideomycetes	Capnodiales	Teratosphaeriaceae	undet.	Teratosphaeriaceae sp.	0.14	1	0	MF942483
15	Dothideomycetes	Capnodiales	undet.	undet.	Capnodiales sp. 1	0.53	1	1	MF942484

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
16	Dothideomycetes	Capnodiales	undet.	undet.	Capnodiales sp. 2	0.40	4	2	MF942485
17	Dothideomycetes	Capnodiales	undet.	undet.	Capnodiales sp. 3	0.37	3	2	MF942486
18	Dothideomycetes	Capnodiales	undet.	undet.	Capnodiales sp. 4	0.15	0	1	MF942487
19	Dothideomycetes	Capnodiales	undet.	undet.	Capnodiales sp. 5	0.62	2	1	MF942488
20	Dothideomycetes	Chaetothyriales	undet.	undet.	Chaetothyriales sp.	0.24	0	1	MF942489
21	Dothideomycetes	Dothideales	Saccotheciaceae	Aureobasidium	*Aureobasidium* sp. 1	0.25	1	4	MF942490
22	Dothideomycetes	Dothideales	Saccotheciaceae	Aureobasidium	*Aureobasidium* sp. 2	0.41	4	1	MF942491
23	Dothideomycetes	Pleosporales	Didymosphaeriaceae	undet.	Didymosphaeriaceae sp.	0.25	2	2	MF942492
24	Dothideomycetes	Pleosporales	Periconiaceae	Periconia	*Periconia* sp.	0.64	1	0	MF942493
25	Dothideomycetes	Pleosporales	Pleosporaceae	Bipolaris	*Bipolaris* sp.	0.12	1	2	MF942494
26	Dothideomycetes	Pleosporales	Pleosporaceae	Curvularia	*Curvularia lunata*	0.12	1	0	MF942495
27	Dothideomycetes	Pleosporales	Pleosporaceae	Edenia	*Edenia gomezpompae*	0.33	1	3	MF942496
28	Dothideomycetes	Pleosporales	Pleosporaceae	Pithomyces	*Pithomyces chartarum*	0.36	2	2	MF942497
29	Dothideomycetes	Pleosporales	Cucurbitariaceae	Pyrenochaetopsis	*Pyrenochaetopsis* sp. 1	1.09	3	5	MF942498
30	Dothideomycetes	Pleosporales	Cucurbitariaceae	Pyrenochaetopsis	*Pyrenochaetopsis* sp. 2	1.50	0	3	MF942499
31	Dothideomycetes	Pleosporales	Thyridariaceae	Roussoella	*Roussoella* sp.	0.37	1	1	MF942500
32	Dothideomycetes	Pleosporales	Teichosporaceae	Teichospora	*Teichospora* sp.	0.26	2	4	MF942501
33	Dothideomycetes	Botryosphaeriales	undet.	undet.	Botryosphaeriales sp.	0.02	0	2	MF942502

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
34	Dothideomycetes	undet.	undet.	undet.	Dothideomycetes sp. 1	0.14	1	3	MF942503
35	Dothideomycetes	undet.	undet.	undet.	Dothideomycetes sp. 2	2.08	5	7	MF942504
36	Eurotiomycetes	Chaetothyriales	Cyphellophoraceae	Cyphellophora	*Cyphellophora eucalypti*	0.62	5	8	MF942505
37	Dothideomycetes	*incertae sedis*	*incertae sedis*	Coniosporium	*Coniosporium* sp.	0.56	1	0	MF942506
38	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	*Exophiala* sp.	0.36	2	2	MF942507
39	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	*Aspergillus* sp. 1	0.01	0	1	MF942508
40	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	*Aspergillus* sp. 2	0.30	0	1	MF942509
41	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium* sp.	0.14	0	2	MF942510
42	Eurotiomycetes	Unclassified Eurotiales.	undet.	undet.	Eurotiales sp.	0.21	3	0	MF942511
43	Leotiomycetes	Rhytismatales	Rhytismataceae	undet.	*Rhytismataceae* sp.	0.12	1	3	MF942512
44	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	Debaryomyces	*Debaryomyces* sp. cf *fabryi*	0.26	3	2	MF942513
45	Sordariomycetes	Calosphaeriales	Pleurostomataceae	Pleurostoma	*Pleurostoma* sp.	0.38	0	2	MF942514
46	Sordariomycetes	*incertae sedis*	Jobellisiaceae	Jobellisia	*Jobellisia guangdongensis*	0.68	0	4	MF942515
47	Sordariomycetes	Glomerellales	Plectosphaerellaceae	undet.	Plectosphaerellaceae sp.	0.14	1	0	MF942516
48	Sordariomycetes	Hypocreales	Cordycipitaceae	Lecanicillium	*Lecanicillium* sp.	0.01	1	0	MF942517

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
49	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma* sp. 1	0.83	0	1	MF942518
50	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma* sp. 2	0.04	1	1	MF942519
51	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium* sp. 2	0.63	2	3	MF942520
52	Sordariomycetes	Hypocreales	Nectriaceae	undet.	Nectriaceae sp. 1	0.06	0	1	MF942521
53	Sordariomycetes	Hypocreales	undet.	undet.	Hypocreales sp.	0.14	1	1	MF942522
54	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	Nigrospora	*Nigrospora oryzae*	0.27	2	4	MF942523
55	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	Nigrospora	*Nigrospora* sp.	3.04	8	8	MF942524
56	Sordariomycetes	Xylariales	Sporocadaceae	Neopestalotiopsis	*Neopestalotiopsis* sp.	0.68	5	7	MF942525
57	Sordariomycetes	Xylariales	Sporocadaceae	Pseudopestalotiopsis	*Pseudopestalotiopsis* sp.	0.02	1	0	MF942526
58	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 1	20.94	9	9	MF942527
59	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 2	9.04	8	9	MF942530
60	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 3	6.98	9	9	MF942531
61	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 4	2.38	9	9	MF942532
62	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 5	2.48	9	9	MF942533
63	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 6	1.24	3	3	MF942534
64	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 7	0.55	2	7	MF942535
65	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 8	0.47	3	3	MF942536
66	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 9	0.68	0	2	MF942537

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
67	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 10	0.67	5	7	MF942528
68	Sordariomycetes	Xylariales	undet.	undet.	Xylariales sp. 11	0.41	1	1	MF942529
	Basidiomycota								
69	Agaricomycetes	Agaricales	Psathyrellaceae	undet.	Psathyrellaceae sp. 2	0.01	0	1	MF942538
70	Agaricomycetes	undet.	undet.	undet.	Agaricomycetes sp. 1	0.02	2	0	MF942562
71	Agaricomycetes	Agaricales	undet.	undet.	Agaricales sp. 1	0.04	0	1	MF942560
72	Agaricomycetes	Agaricales	undet.	undet.	Agaricales sp. 2	0.01	0	1	MF942539
73	Agaricomycetes	Boletales	Coniophoraceae	Coniophora	*Coniophora* sp.	0.02	1	0	MF942540
74	Agaricomycetes	Corticiales	Corticiaceae	Sistotrema	*Sistotrema* sp.	0.02	0	1	MF942541
75	Agaricomycetes	Corticiales	Corticiaceae	undet.	Corticiaceae sp.	0.04	0	1	MF942542
76	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Phellinus	*Phellinus* sp. cf. *resupinatus*	0.55	2	3	MF942544
77	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma	*Ganoderma* sp. 1	0.09	1	1	MF942545
78	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma	*Ganoderma* sp. 2	0.02	1	0	MF942546
79	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma	*Ganoderma* sp. 3	0.04	1	0	MF942547
80	Agaricomycetes	Polyporales	Irpicaceae	undet.	Irpicaceae sp.	0.05	1	0	MF942556
81	Agaricomycetes	Polyporales	Lentinaceae	undet.	Lentinaceae sp.	0.02	1	1	MF942548
82	Agaricomycetes	Polyporales	Meruliaceae	Cabalodontia	*Cabalodontia* sp.	0.11	1	0	MF942549

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
83	Agaricomycetes	Polyporales	Meruliaceae	Junghuhnia	*Junghuhnia* sp.	0.01	0	1	MF942551
84	Agaricomycetes	Polyporales	Phanerochaetaceae	Pseudolagarobasidium	*Pseudolagarobasidium acaciicola*	0.20	0	2	MF942552
85	Agaricomycetes	Polyporales	Phanerochaetaceae	undet.	Phanerochaetaceae sp.	0.01	1	0	MF942561
86	Agaricomycetes	Polyporales	Polyporaceae	undet.	Polyporaceae sp. 3	0.01	1	0	MF942553
87	Agaricomycetes	Polyporales	Polyporaceae	undet.	Polyporaceae sp. 4	0.02	1	0	MF942554
88	Agaricomycetes	Polyporales	Polyporaceae	Trametes	*Trametes versicolor*	0.12	3	1	MF942555
89	Agaricomycetes	Polyporales	Meruliaceae	undet.	Meruliaceae sp.	0.01	1	0	MF942550
90	Agaricomycetes	Russulales	Peniophoraceae	Peniophora	*Peniophora* sp.	0.01	1	0	MF942557
91	Agaricomycetes	Russulales	Stereaceae	Stereum	*Stereum* sp.	0.02	1	0	MF942558
92	Agaricomycetes	Thelephorales	Thelephoraceae	Pseudotomentella	*Pseudotomentella larsenii*	0.02	1	0	MF942559
93	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Erythrobasidium	*Erythrobasidium* sp.	0.01	0	1	MF942563
94	Cystobasidiomycetes	Sporidiobolales	Sporidiobolaceae	Rhodosporidiobolus	*Rhodosporidiobolus ruineniae*	0.06	1	1	MF942564
95	Exobasidiomycetes	Exobasidiales	Brachybasidiaceae	Meira	*Meira cf. argovae*	0.04	0	3	MF942565
96	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	Rhodotorula	*Rhodotorula* sp. 1	0.37	3	1	MF942566
97	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	Rhodotorula	*Rhodotorula* sp. 2	0.02	1	0	MF942567

No.	Class	Order	Family	Genus	Species	454 sequencing			Genbank accession #
						% read	#Dis	#Rot	
98	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	Tausonia	*Tausonia pullulans*	0.14	2	1	MF942568
99	Tremellomycetes	Filobasidiales	Filobasidiaceae	Filobasidium	*Filobasidium* sp. 1	0.05	1	1	MF942569
100	Tremellomycetes	Filobasidiales	Filobasidiaceae	Filobasidium	*Filobasidium* sp. 2	0.01	1	0	MF942570
101	Tremellomycetes	Filobasidiales	Filobasidiaceae	Naganishia	*Naganishia* sp.	0.02	0	2	MF942571
102	Tremellomycetes	Tremellales	Bulleribasidiaceae	Hannaella	*Hannaella luteola*	0.16	2	4	MF942572
103	Tremellomycetes	Tremellales	Bulleribasidiaceae	Hannaella	*Hannaella pagnoccae*	0.06	1	2	MF942573
104	Tremellomycetes	Tremellales	Rhynchogastremataceae	Papiliotrema	*Papiliotrema* sp. 1	0.09	1	1	MF942574
105	Tremellomycetes	Tremellales	Rhynchogastremataceae	Papiliotrema	*Papiliotrema* sp. 2	0.24	0	5	MF942575
106	Tremellomycetes	Tremellales	Rhynchogastremataceae	Papiliotrema	*Papiliotrema* sp. 3	0.09	1	3	MF942576
107	Tremellomycetes	Tremellales	Rhynchogastremataceae	Papiliotrema	*Papiliotrema* sp. 4	0.06	0	2	MF942577
108	Tremellomycetes	Tremellales	Trimorphomycetaceae	Saitozyma	*Saitozyma podzolica*	0.20	2	0	MF942578
109	Tremellomycetes	Tremellales	Trimorphomycetaceae	Saitozyma	*Saitozyma* sp.	0.52	2	4	MF942579

Abbreviations: % reads: percentage of overall reads that were associated to this species; #Dis: number of pooled samples from discoloured wood in which this species was found; #Rot: number of pooled samples from rotten wood in which this species was found.

Appendix 4-3: Potential wood-rotting basidiomycetes associated with discoloration or decay

No.	Species	454 sequencing		
		% reads	#Dis	#Rot
1	*Cabalodontia* sp.	0.11	1	0
2	Irpicaceae sp.	0.05	1	0
3	*Ganoderma* sp. 3	0.04	1	0
4	*Coniophora* sp.	0.02	1	0
5	*Ganoderma* sp. 2	0.02	1	0
6	Polyporaceae sp. 4	0.02	1	0
7	*Stereum* sp.	0.02	1	0
8	*Pseudotomentella larsenii*	0.02	1	0
9	Agaricomycetes sp. 1	0.02	2	0
10	Meruliaceae sp.	0.01	1	0
11	Polyporaceae sp. 3	0.01	1	0
12	*Peniophora* sp.	0.01	1	0
13	Phanerochaetaceae sp.	0.01	1	0
14	*Pseudolagarobasidium acaciicola*	0.20	0	2
15	Corticiaceae sp.	0.04	0	1
16	Agaricales sp. 2	0.04	0	1
17	*Sistotrema* sp.	0.02	0	1
18	Psathyrellaceae sp. 2	0.01	0	1
19	Agaricales sp. 3	0.01	0	1
20	*Junghuhnia* sp.	0.01	0	1

Abbreviations: % reads: percentage of overall reads that were associated to this species; #Dis: number of pooled samples from discoloured wood in which this species was found; #Rot: number of pooled samples from rotten wood in which this species was found.

Appendix 4-4: OTUs detected by isolation from both *Acacia* hybrid plantations at Nghia Trung and Phan Truong Hai in Vietnam or by 454 environmental sequencing at Phan Truong Hai and isolation from one or both sites.

No.	Class	Order	Family	Genus	Species	Isolates obtained at		454 sequencing		
						NT	PTH	% reads	#Dis	#Rot
	Ascomycota									
1	Dothideomycetes	Unclassified Botryosphaeriales	undet.	undet.	Botryosphaeriales sp.		1	0.02	0	2
2	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	Debaromyces	*Debaromyces* sp. cf *fabryi*		1	0.26	3	2
3	Sordariomycetes	Glomerellales	Glomerellaceae	Colletotrichum	*Colletotrichum* sp.	1	1	-		
4	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	*Clonostachys* sp. 1	8	1	-		
5	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma* sp. 1	2		0.83	0	1
6	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma* sp. 2	1		0.04	1	1
7	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium* sp. 2	1		0.63	2	3
8	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium* sp. 3	4	3	-		
9	Sordariomycetes	Hypocreales	Nectriaceae	undet.	Nectriaceae sp. 1	2		0.06	0	1
10	Sordariomycetes	Microascales	Ceratocystidaceae	Ceratocystis	*Ceratocystis manginecans*	4	2	-		
11	Sordariomycetes	Xylariales	Sporocadaceae	Neopestalotiopsis	*Neopestalotiopsis* sp.	1	10	0.68	5	7

No.	Class	Order	Family	Genus	Species	Isolates obtained at		454 sequencing		
						NT	PTH	% reads	#Dis	#Rot
12	Sordariomycetes	Xylariales	Sporocadaceae	Pestalotiopsis	*Pestalotiopsis* sp. 2	11	4	-		
13	Sordariomycetes	Xylariales	Sporocadaceae	Pseudopestalotiopsis	*Pseudopestalotiopsis* sp.	29	19	0.02	1	0
	Basidiomycota									
14	Agaricomycetes	Agaricales	Schizophyllaceae	Schizophyllum	*Schizophyllum commune*	3	6	-		
15	Agaricomycetes	Polyporales	Phanerochaetaceae	Pseudolagarobasidium	*Pseudolagarobasidium acaciicola*	2	2	0.20	0	2
16	Agaricomycetes	Polyporales	Polyporaceae	undet.	Polyporaceae sp. 2	9	1	-		
17	Agaricomycetes	Polyporales	undet.	undet.	Polyporales sp. 1	5	1	-		

Abbreviations: NT = Nghia Trung; PTH = Phan Truong Hai; % reads: percentage of overall reads that were associated to this species; #Dis: number of pooled samples from discoloured wood in which this species was found; #Rot: number of pooled samples from rotten wood in which this species was found.

Appendix 5-1: Summary of a two-way ANOVA that examined the effects of nine *Acacia* genotypes and two controls (mock wounded and unwounded control trees) on concentrations of phenolic compounds. N = 4 replicates of each combination of tree.

Phenolic compounds*	*Acacia* genotypes *p*-value	Mock wounded vs. unwounded *p*-value	*Acacia* genotype x wounded control *p*-value
Total peaks	0.01	<0.001	0.12
Condensed tannins	<0.001	0.26	0.11
Cp1	<0.001	0.002	0.13
Cp2	0.04	<0.001	0.06
Cp3	0.79	<0.001	0.66
Cp4	0.01	<0.001	0.08
Cp5	<0.001	<0.001	0.15
Cp6	<0.001	0.002	0.31
Cp7	0.01	0.004	0.28
Cp8	0.003	<0.001	0.07

*See Table 5-4 for details of phenolic compounds Cp1 – 8.

Appendix 5-2: Effects of nine *Acacia* genotypes on phenolic compounds. Entries are concentrations (µg/mL) of phenolic compounds averaged over mock wounded and unwounded control trees (N = 8 trees). Means with different letters in the same row are significantly different ($p < .05$).

Phenolic compounds*	*A. auriculiformis*		*Acacia* hybrid						*A. mangium*
	AA1	AA9	AH1	AH7	BV10	BV33	TB12	TB6	AM
Total peaks	71.8^{ab}	87.3^{bc}	70.9^{ab}	103.0^{c}	97.2^{c}	77.9^{abc}	71.4^{ab}	61.7^{a}	67.1^{ab}
Condensed tannins	353.0^{cd}	404.5^{d}	269.4^{ab}	300.3^{bc}	327.1^{bcd}	263.2^{ab}	224.9^{a}	211.8^{a}	296.2^{bc}
Cp1	2.3^{d}	2.4^{d}	3.9^{e}	1.71^{bcd}	1.4^{bc}	0.8^{a}	2.13^{cd}	1.12^{ab}	1.77^{bcd}
Cp2	0.3^{a}	0.2^{a}	0.4^{a}	1.3^{b}	0.4^{a}	0.5^{a}	0.5^{a}	0.5^{a}	0.7^{ab}

Phenolic compounds*	*A. auriculiformis*		*Acacia* hybrid						*A. mangium*
	AA1	AA9	AH1	AH7	BV10	BV33	TB12	TB6	AM
Cp3	0.5^{a}	0.7^{a}	0.4^{a}	0.9^{a}	0.5^{a}	0.6^{a}	0.7^{a}	0.4^{a}	0.6^{a}
Cp4	1.3^{a}	1.9^{ab}	2.2^{abc}	3.2^{bcd}	3.5^{cd}	2.3^{abc}	2.2^{abc}	2.5^{bcd}	4.0^{d}
Cp5	2.8^{de}	3.4^{e}	1.8^{bcd}	2.6^{de}	2.9^{de}	0.7^{a}	2.1^{cde}	0.9^{ab}	1.1^{abc}
Cp6	0.6^{a}	0.4^{a}	3.1^{cd}	5.4^{d}	2.1^{bc}	2.2^{bc}	2.5^{bc}	1.4^{ab}	2.8^{bc}
Cp7	2.8^{bc}	3.2^{c}	1.9^{ab}	2.7^{bc}	3.2^{c}	1.6^{a}	2.3^{abc}	1.8^{ab}	1.9^{ab}
Cp8	18.1^{abc}	22.1^{bcde}	24.3^{cde}	26.3^{de}	30.9^{e}	24.3^{cde}	21.5^{bcd}	16.8^{ab}	14.7^{a}

*See Table 5-1 and Table 5-4 for details of *Acacia* genotypes and phenolic compounds Cp1 – 8, respectively.

Appendix 5-3: Effects of wounding on concentrations of phenolic compounds. Entries are concentrations (µg/mL) of phenolic compounds averaged over mock wounded and unwounded control trees (N = 36 trees). Means with the same letter in the same row are not significantly different ($p < .05$).

Phenolic compounds*	Mock wounded	Unwounded
Total peaks	99.5[a]	60.5[b]
Condensed tannins	298.9[a]	279.5[a]
Cp1	2.2[a]	1.5[b]
Cp2	1.3[a]	0.1[b]
Cp3	1.1[a]	0.2[b]
Cp4	4.0[a]	1.4[b]
Cp5	2.5[a]	1.4[b]
Cp6	2.6[a]	1.4[b]
Cp7	2.7[a]	2.0[b]
Cp8	29.0[a]	16.1[b]

*See Table 5-4 for details of phenolic compounds Cp1 – 8.